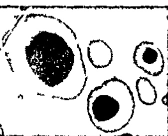
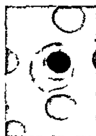
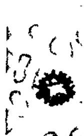
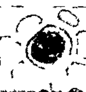
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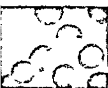
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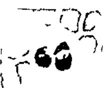










Ea ly t med te nd ate megal blast (perpic ous na m a)

Intermediate megaloblast with Howell Jolly body (per niciousana m)

Later megaloblast w th th ee Howell-Jolly bod s (pernicious an em a)

Megaloblast in metapha e (pernic ous anaemia)

rly normoblast

Intermed ate normoblast

Late intermedi te normoblast (iron deficiency anaemia)

Twinning deformity intermediate normoblast (haemolytic anaemia)

Leuka m c monocyte

L.E. cell

H inz bodies (Phe ac tin poison g—m thyl violet and eos n)

Feulgen appearance of micro-myeloblasts

P ppenh m r bod es lymphosarcoma)

Mononu l ar of gla dul r fe er

Plasm cell (my loma)

Cabot ring (pernicious anaemia)

Leuk mic myelobla t

Ba oph l c st ppl ng (lead po on g)

Hypersegmented ne troph l (pernic ous na mia)

Nuclear appendages in neutrophil (type found in both sexes more common in males)

Meg k ryocyte

HANDBOOK OF

HAEMATOLOGICAL AND BLOOD TRANSFUSION TECHNIQUE

J W DELANEY, FIMLT

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1960

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HANDBOOK OF HISTOPATHOLOGICAL TECHNIQUE

C F A CULLING FIMLT FRMS

AN INTRODUCTION TO MEDICAL LABORATORY TECHNOLOGY (2nd Edition)

F J BAKER FIMLT FRMS
R E SILVERTON AIMLT FRMS
EVELINE D LUCKCOCK AIMLT

MEDICAL LABORATORY INVESTIGATIONS

IAN DAWSON MA MD MRCP
WILLIAM GOLDIE MA MB FRCP FRCP(E)


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FOREWORD

A LABORATORY is as good as its technical staff One of the principal tasks of chief and senior technicians is to see that junior technicians *are properly trained so that their benchwork is accurate and reliable* and they reach the standards required at the various examinations they have to take Mr Delaney is well known for his clear and concise teaching in the lecture room and at the bench, and many technicians have reason to be grateful to him He has now collected in book form the essence of current teaching on haematology and blood transfusion and has set it out in such a way that it will prove valuable both to those engaged in practical work in the laboratory and to those working for examinations

In these days when so many books tend to be compilations of the views of other workers it is refreshing to find one whose author has clearly a great deal of practical experience behind his writing It should prove of great value and I wish it every success

IAN DAWSON

PREFACE

It would not be possible to compress into a volume of this size all the accumulated knowledge of haematology and blood transfusion Instead I have tried to produce a book which will assist candidates to pass the examination of the I M L T, but with the best book in the world I do feel that much bench work and a qualified instructor are also essential for examination success

It is certain that by the time this book is published some sections will be out of date but that is inevitable in a subject of which our knowledge increases daily I have tried therefore to teach basic techniques and make no apology for the length of Chapter 1 which I feel is essential for the understanding of the aetiology of blood disorders nor for the inclusion of Chapter 7, which is an introduction to the study of genetics The chapter dealing with statistics is necessarily incomplete because of the nature of the subject but I hope it will stimulate the reader to further study

I must thank Dr J Humble for kindly reading the first section of this book his chief technician, Mr N Thacker F I M L T for valuable advice and reading the proofs and Dr J P Nicholson for reading the chapter on radioisotopes Mr L Marsh read the second section and provided many useful suggestions as did Dr K L Goldsmith and I am grateful to them both My thanks must also go to Dr W Bloom for permission to reproduce Fig 2 from *A Textbook of Histology* by A M Maximow and W Bloom to Professor D D Van Slyke for kindly allowing me to use his technique and line chart for the copper sulphate haemoglobin method to Professor L J Witts for allowing me to use his classification of the megaloblastic anaemias and to Dr G I M Ross for much of the B_1 material and I am also grateful to Dr Rosemary Biggs for permission to reproduce graphs from her works on coagulation If I have failed to give credit in the text for any technique or original hypothesis I must apologize in advance

The photomicrographs are due to Mr R Sandison F I M L T and Mr E Pittock and the other photographic work was done by the Westminster Medical Photographic Unit Only I can take the blame for the drawings Last but not least I must thank my sister Mrs A Davis and Miss B Southey, who so patiently typed and retyped the manuscript and I hope learned some haematology while doing so

J D

CONTENTS

SECTION I

HAEMATOLOGY

SECTION II

BLOOD TRANSFUSION TECHNIQUE

SECTION I

HAEMATOLOGY

CHAPTER I

THE ORIGIN, LIFE AND DEATH OF THE BLOOD CELLS

EMBRYOLOGICAL BLOOD FORMATION

THE STUDY of human embryological blood formation is hindered by the lack of material It is therefore necessary to study the processes as they occur in the chick and compare them with those known to occur in man In this way gaps in the understanding of these processes may be filled by reasonable assumptions The following description will be termed embryological blood formation without reference to animal or species

THE OVUM FERTILIZATION

The mature ovum is fertilized usually in the fallopian tube Immediately the two pro nuclei—represented by the ovum nucleus and the head of the sperm—have fused, segmentation of the fused mass begins By the time the ovum has reached the uterus it consists of a cyst like structure containing a mass of cells and is called the morula (Fig 1 (*a*)) The cells which form the outer wall of the cyst exert a lytic action on the lining of the uterus, and thus a nest is excavated for the ovum The uterine membrane then grows over the ovum sealing it away from harm The hormones which make this implantation possible are not within the province of this book and may be conveniently ignored

STRUCTURE OF THE EMBRYO

The embryo at this stage may be represented diagrammatically as a sphere enclosing two smaller spheres (Fig 1 (*b*)) The outer cyst like structure is the chorion, the inner two are the amnion and the yolk sac The cells forming the amnion are known as ectoderm, and those of the yolk sac as entoderm Where these two structures touch a third layer of cells arises from the ectoderm and is called mesoderm (Figs 1 (*c*) and (*d*)) The mesoderm is laid down between ectoderm and entoderm and by numerous processes eventually forms the connective tissues of the body The area of the yolk sac outside the point of contact of the two primary layers is called the area vasculosa

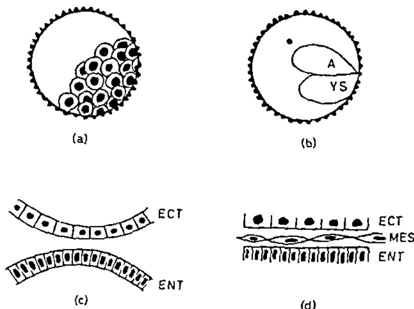


FIG 1 —Diagrammatic representation of cells (*a*) Morula stage (*b*) three-cyst stage—A amnion Y S yolk sac (*c*) primary layers—ECT ectoderm ENT entoderm (*d*) mesoderm arising between ectoderm and entoderm

MESOBLASTIC STAGE OF BLOOD FORMATION

At this stage blood formation begins and is known as the meso blastic stage Mesodermal cells migrate out to the area vasculosa of the yolk sac and form collections of cells called blood islands The mesoderm proper is a syncytium that is a sheet of nuclei with no obvious cell boundaries and it forms the body mesenchyme which is the forerunner of the connective tissues The syncytial mesoderm by a hollowing out process forms tubes one cell thick which are primitive blood vessels They become filled with primitive blood plasma secreted by the cells lining the walls Some of the cells which take part in the hollowing out process float away in the plasma as the first blood cells Individual cells lining the vessels also sometimes break off and float away and become blood cells (Fig 2) The cells now forming the vessels no longer exist as a syncytium cell membranes having appeared but have differentiated to become primitive endothelium They retain the power to differentiate to perform other functions The nucleated cells which are now present *in the plasma are termed the megaloblasts of Ehrlich (Fig 3) and* although having the same name as cells seen in pernicious and other

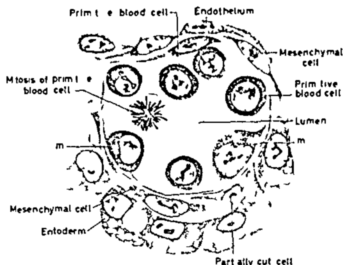


FIG 2 —Cross section of a vessel of the area vasculosa of a rabbit embryo 8½ days m rounding off of endothelial cells and their transformation into primitive blood cells (*Reproduced by courtesy of the authors and publishers* (*Saunders Philadelphia*) *of A Textbook of Histology* *4th edn* 1942)

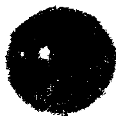


FIG 3 —Megaloblast of Ehrlich (May Grunwald Giemsa) a normal size red cell is shown for comparison (× 750)

anaemias they are not pathological but embryological cells All the cells of the embryo at this stage are rapidly dividing and differentiating but true blood cells as known, have not yet appeared The cells described as megaloblasts have differentiated purely to perform the essential work of carrying oxygen to the tissues and to this end have built up a primitive haemoglobin from precursor substances already present in their cytoplasm However they have differentiated only to do this work and as soon as more suitable cells have been developed they disappear The primitive white cells are developed from the mesenchyme cells at this same stage of development These cells which are recognizable as leucocytes make their way to the vessels and pass through the interstices between the endothelial cells thus entering the vessels

HEPATO SPLENIC STAGE OF BLOOD FORMATION

The growing embryo (Fig 4) needs more and more blood as development proceeds Mesenchymal cells are found in the primitive liver spleen thymus and other sites so that at about 6–8 weeks of foetal life the hepatosplenic stage of blood formation has begun (Fig 5) The primitive stem cells are still producing blood cells but in different sites

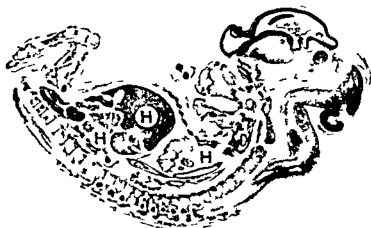


FIG 4—Section of 11 week human embryo (H_2E) H Sites of haemopoiesis thymus liver and spleen

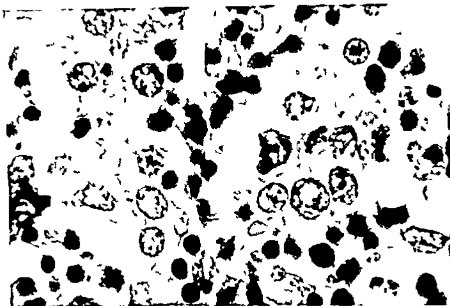


FIG 5—High power view of liver from embryo shown in Fig 4 (H and E) islands of haemopoiesis are seen between the large parenchymal cells (× 425)

MYELOID STAGE OF BLOOD FORMATION

At about middle foetal life the bone marrow is formed and the myeloid stage of blood formation is begun to continue throughout adult life The mesenchymal cells however although their activity is restricted are still present in the other sites and given a stimulus will resume their embryonic function Thus if a newborn child loses blood rapidly and continuously either from haemorrhage or haemolysis areas of new blood formation reform in the liver spleen muscle subcutaneous tissue or in any area where there is a mesenchymal rest Histological sections of such tissue show very active centres of haemopoiesis They are referred to as ectopic or heterotopic areas of bone marrow In blood diseases such as leukaemia nodules of the characteristic cells appear in the skin This may not be an invasion of tissue like a cancerous growth but the stimulus which has been applied to the white cell production centres operates equally well on the rest of the persisting mesenchymal cells scattered throughout the body no matter where they are The stimulus causes the stem cells to differentiate rapidly so that a nodule of leucocytes is produced Again in the disease myelosclerosis the bone marrow becomes replaced with fibres and

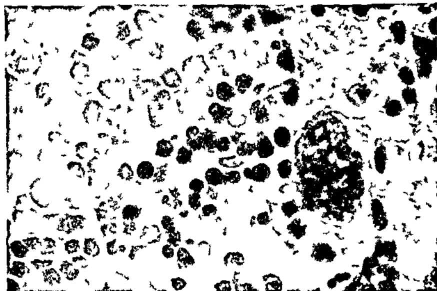


FIG 6 —Section of spleen from a case of myelosclerosis (H and E) haemopoietic activity includes megakaryocytic production (× 425)

the spleen and liver revert to their embryonic function of producing the cells the marrow is failing to manufacture Sections of such tissue show active haemopoiesis (Fig 6)

BLOOD FORMATION IN THE ADULT

Undifferentiated mesenchyme still exists in the adult as a syncytium scattered through the body On appropriate stimulation cell boundaries appear and fine argyrophil fibrils running through the cytoplasm can be demonstrated These cells were first called by Aschoff the reticulo endothelial system This is a system of cells found in many organs and performing many vital physiological functions The system consists of cells which are phagocytic and dye storing in a reticulum of silver salt reducing fibres Some of the cells may become freed from the reticulum and are then known as wandering histiocytes macrophages or clasmatocytes The resemblance of such cells to the monocyte of the blood is striking enough to assume a direct descent of the monocyte from the reticulum cell and indeed some authorities hold this or a similar view The freed reticulum cell may become a fibroblast or littoral cell but the concept that cells of the mesenchyme are the ultimate precursor still existing in the adult body gives a wider system which includes Aschoff s reticulo endothelial system and is known as the reticular or lymphoreticular system of Maximow

THE POLYPHYLETIC AND ALTERNATIVE THEORIES

Thus far the origin of the blood cells is not in much doubt, but a great conflict of view exists regarding the processes as they occur from the mesenchymal cell The most attractive theory is the complete polyphyletic theory which postulates a primary cell the haemocytoblast which will develop into a series of blast cells myeloblast lymphoblast monoblast erythroblast and megakaryo blast These cells in turn by division and maturation will pass through a pro-cyte or pre cyte stage and then on to the adult cell The haemocytoblast is a cell whose morphological characteristics vary from authority to authority, but probably the best criterion of identification is the personal conviction of the observer that the cell although at that moment not an obvious cell of the blood, could develop into either a red or white cell

An alternative theory postulates that the lymphocyte of lymphatic tissue is the primitive blood cell and can form all the other blood cells The apparent lack of function of the lymphocyte lends some support to the theory and some evidence does exist that the large lymphocyte is more mature than the small Lymphocytes are found in all the lymphoid tissues of the body, the spleen thymus tonsils Peyer s patches of the gut and lymph nodes and also in the bone marrow although normally only in relatively small numbers in adults The apparent production of lymphocytes can be seen in any cut section of a lymph gland, when the germinal centres are examined These centres consist of a central pale area of cells in mitosis (which may be lymphoblasts developing from mesenchyme) surrounded by a zone of large lymphocytes with an outer zone of many small lymphocytes The presence of small lymphocytes in the bone marrow needs some explanation and two theories are put forward to account for this One is that the cells are actually produced in lymphatics and enter the bone marrow where they either pass into the blood stream disintegrate or act as precursors for cells of the myeloid series Alternatively the lymphocytes may be actually produced in the bone marrow in specific centres and such marrow foci have been often described

The polyphyletic theory is also subject to objections so that it is only held in part by some authorities Three or four blast cells only may be postulated for example the myeloblast being regarded as the precursor of the myeloid series the monocytes and the megakaryocytes, the lymphoblast as the ancestor of the lymphocytes and the erythroblast as the red cell precursor The monocyte according to one school is produced in the liver and spleen from reticulum cells

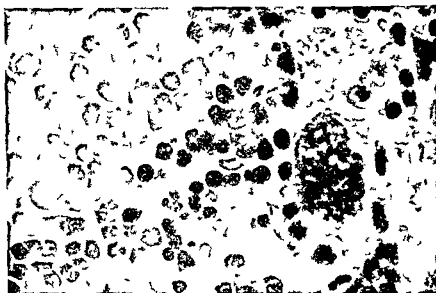


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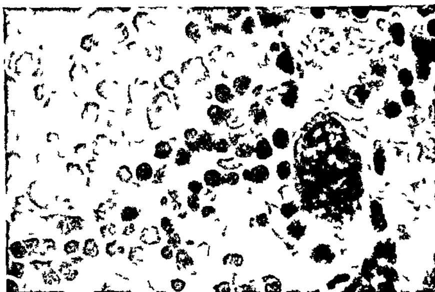


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MORPHOLOGY OF THE BLOOD CELLS

The descriptions of blood and marrow cells which follow assume the positive identification of a blast cell precursor for each of the blood elements It should be noted however that such cells are not capable of easy distinction and in many cases must be identified by the company they keep It must also be realized that the stages of development described are arbitrary there being a gradual transition rather than abrupt change in the later cells The staining appearances are those seen with Romanowsky stains

THE RED CELL SERIES

The pro erythroblast is a large cell (about 16μ in diameter) round or ovoid with deeply staining cytoplasm The nucleus is large central or slightly eccentric and the chromatin tends to be chunky although this may not be obvious in the very early cell There are one to four nucleoli which are often prominent This cell under normal circumstances divides to form early normoblasts which are slightly smaller but still with deeply staining cytoplasm The chromatin has aggregated further and the nucleoli have disappeared The early normoblast also divides to produce intermediate normoblasts in which the cytoplasm stains lighter owing to the presence of haemoglobin which is being built up within the cell The nucleus is smaller and the chromatin has aggregated to give the nucleus a cartwheel appearance This cell by maturation and condensation forms a late normoblast a still smaller cell with fully haemoglobinized cytoplasm and a black pyknotic nucleus which may be central or eccentric The nucleus is either extruded whole or is broken into fragments by a process termed karyorrhexis and these particles are then extruded in their turn When the nucleus disappears the cell is apparently a normal red cell although it may show staining abnormalities Special staining methods however reveal a filamentous structure which may be a fine reticulum or a coarse network and possibly represents a nuclear framework Such a cell is still not mature and is called a reticulocyte With the disappearance of the reticulum the cell is seen as a fully haemoglobinized biconcave disc with no cytoplasmic inclusions and is termed a normocyte The term erythron is used when the red cell and its precursors are regarded as a tissue Normally nucleated red cells are not seen in the peripheral blood the only evidence of immaturity being a very small percentage (1–2 per cent) of reticulocytes

THE MYELOID SERIES

The myeloblast is often indistinguishable from other blast cells but later stages may develop a few rods or dots of purplish red material known as Auer bodies in the cytoplasm The nucleoli number one to four and are usually indistinct being described as less dense areas of nucleoplasm The pro myelocyte is produced by division from the myeloblast and is larger than the parent cell The cytoplasm is not so deeply stained and shows a few granules of different colours with Romanowsky stains The nucleus is round and nucleoli are very indistinct or more often absent From the pro myelocyte is formed the myelocyte which contains within its cytoplasm numerous granules which stain in a characteristic manner The eosinophil myelocyte has large red granules which often appear as red circles the basophil large deep black granules and the neutrophil myelocyte fine faint pink granules

After this stage is reached the nucleus becomes indented and kidney shaped and is known as a metamyelocyte The nucleus undergoes further changes with maturation becomes elongated and twisted and is then a staff cell Finally the nucleus becomes lobulated and as the cell ages the number of lobes increases to five or six or even more The cell is then a fully mature granulocyte The polymorphonuclear eosinophil does not usually become very lobulated having only two or three lobes whilst the basophil often has its nucleus completely hidden by the large black granules of the cytoplasm Normally maturation of this series occurs in the marrow the earliest cell seen in the blood being the staff cell

THE LYMPHOID SERIES

The lymphoblast is in many cases indistinguishable morphologically from other blast cells although some authorities stress that the nucleoli are so sharp in outline as to give the impression of having nucleolar membranes The pro lymphocyte is smaller but traces of nucleoli may remain and the chromatin is loosely arranged The cytoplasm still stains deeply but is hyaline and transparent The mature lymphocyte is a small round cell with scanty deeply staining cytoplasm often showing an unstained perinuclear zone The large lymphocyte—regarded by some authorities as the more mature cell—possesses more cytoplasm and often a few granules staining bright blue Normally lymphoblasts and pro lymphocytes are not seen in the blood stream

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THE BLOOD FORMING ORGANS

BONE MARROW

Bone marrow in the adult amounts to about 4 per cent of body weight and is either red and active or yellow and fatty Under pathological conditions it may have a greyish appearance looking like pus The bones of an infant up to 5–7 years of age are entirely occupied by red marrow but as age advances the long bones become inactive until in the twenties only the flat bones ribs and upper ends of the femur and humerus contain red marrow In the normal adult weighing 75 kg the red marrow occupies 1 200–1,500 ml and contains 970 $\times$ 10^9 cells The rest of the marrow is yellow and fatty but can still be called on as a reserve After 70 years of age even the rib marrow becomes fatty Since the infant has no reserve of fatty marrow severe haemorrhage or haemolysis results in the formation of extramedullary centres of haemopoiesis which arise in those organs which were producing blood cells in embryonic life

Structure and function of bone marrow

Microscopically the marrow consists of a series of thin walled vessels called sinusoids connected to the nutrient artery and other blood vessels of the bone and many free cells and reticulum fibres Depending on the activity of the marrow there is a varying amount of fat and a few lymphoid follicles are present but no lymph vessels Red marrow shows numerous red and white cells in varying stages of development Yellow marrow shows only scanty red and white cells and some fat Should the marrow be greyish it will be found to be crowded with leucocyte elements The sinusoids are composed of littoral cells which are flattened and fixed macrophages derived from persisting mesenchyme The finer vessels are termed inter sinusoidal capillaries and are often collapsed on themselves so that only a potential space exists between the walls The red cell series is produced in the collapsed intersinusoidal capillaries by prolifera tion of the primitive cells lining the vessels When the capillary opens the cells are washed into the larger sinusoids where they mature and finally pass into the general circulation The leucocytes are produced extravascularly and migrate to the sinusoids and thence to the peripheral blood It is not clear at what stage these elements enter the sinusoids but under normal circumstances some mechanism probably hormonal prevents the entrance of any but mature cells into the blood stream The dissolution of the cytoplasm of the megakaryocytes may be helped mechanically by the flow of plasma in the sinusoids to form platelets

THE MONOCYTE SERIES

Morphologically the monoblast is identical with other blast cells but may show a somewhat irregular outline and one very large nucleolus often with a small one The pro monocyte is a similar cell but the cytoplasm is muddy and the nuclear chromatin has a tendency to aggregate in a rope like manner The mature monocyte is a large cell often having an irregular outline The cytoplasm stains lightly has a hyaline appearance and contains numerous sky blue granules The nucleus is round or horse shoe shaped and the chromatin is definitely rope like in appearance The mature monocyte is the only cell of this series normally seen in the peripheral blood

THE PLASMA CELL SERIES

The plasmablast is distinguished from other blast cells only by the cells with which it is associated The pro plasmacyte tends to be ovoid has a royal blue cytoplasm and an eccentric nucleus with traces of one or more nucleoli The plasma cell proper is ovoid with a sky blue cytoplasm, often vacuolated and the nuclear chro matin is aggregated radially to form the familiar cartwheel nucleus Turk s irritation cells would seem morphologically to belong to this series although what are often called Turk cells may in fact be pro plasmacytes which often appear in the blood stream especially in children Mature plasma cells are rarely seen in the peripheral blood The Turk cell is described as ovoid with deeply staining cytoplasm often vacuolated and an eccentric condensed nucleus

THE PLATELET SERIES

The megakaryoblast is reported as a very large cell (about 40μ in diameter) with a convoluted nucleus and no obvious nucleoli The pro megakaryocyte is a similar cell but the cytoplasm contains a few granules The adult megakaryocyte has an irregular outline *and exhibits blunt pseudopodia* The cytoplasm contains numerous sky blue granules and the nucleus is large and convoluted Platelets are produced by budding off from the pseudopodia or by actual dissolution of the cytoplasm In effect the platelet—which is a round or stellate piece of material 1–2μ in diameter—is part of the megakaryocyte cytoplasm Baby megakaryocytes capable of amoeboid movement and phagocytic activity may be seen in normal marrow and in pathological states may even enter the blood stream Normally only the platelet is seen in peripheral blood

blood elements lymphocytes and monocytes predominating over granulocytes Numbers of red cells platelets and free macrophages are also present The red pulp is associated with venous branches whereas the white pulp which is true lymphoid tissue follows the course of the arteries The spleen has many functions among which is the production of lymphocytes in common with the other lymphoid tissues and it would seem to be a centre for the production of monocytes The macrophages ingest effete red cell fragments and platelets blood debris and bacteria Excess lipoid is removed by these cells and they are seen loaded with lipoid material in such disorders as Gaucher s disease and diabetic lipaemia The spleen stores iron resulting from the breakdown of haemoglobin and hoards red cells and leucocytes in some conditions The organ has also a hormonal function regulating the release of certain marrow cells This inhibitory effect on granulocytes may be quite marked The destruction of effete red cells may be hastened by the amount of lysolecithin in the spleen since this organ has a high concentration of lecithinase

Effects of splenectomy

Splenectomy is associated with an increase in the numbers of circulating lymphocytes eosinophils and platelets Its functions are then assumed by the liver lymph glands and other organs after a short lag period The spleen may be accompanied in some conditions by a series of small accessory spleens any one of which may attain near normal size after removal of the main organ

THE DEATH AND DISPOSAL OF THE BLOOD CELLS

THE RED CELL

The red cell a biconcave disc with no nucleus liberated into the blood stream is mainly concerned with the transport of oxygen Its shape is such that it can squeeze into the finest capillaries its surface area is large enough for the complicated chemical and physical reactions which take place during its functions It is inevitable however that the wear and tear on a cell which has no life *per se* will be considerable The life of the average red cell determined by methods dealt with in a later chapter is less than 4 months At the end of this time the cell becomes effete and worn out tending to pear shape with a long point This projection actually seems to shake itself free and the process is repeated until the cell is literally fragmented The pieces are ingested by cells of the

Reaction of bone marrow to stimuli

In health the numbers of cells and the ratio of red to white cells are remarkably constant being only sufficient to replace normal wastage and increases needed by bodily growth The marrow however responds immediately to stimulus whether natural or pathological and to a great extent the reaction is specific Should the stimulus be haemorrhage or exaggerated red cell destruction fresh capillaries appear in the fatty marrow These are visible macroscopically and appear as a fine red network Microscopically the active marrow shows a disturbance of the leucocyte red cell ratio with numerous normoblasts in various stages and a lesser increase in granular cell precursors Such a condition is termed erythroid hyperplasia and is characterized by the appearance of large red cells in the peripheral blood These cells are an attempt on the part of the marrow to make up the losses by providing a larger vehicle for the transport of oxygen The increase of leuco poiesis in haemorrhage is termed sympathetic hyperplasia Repeated haemorrhage or the action of some poisons may result in the marrow becoming depressed so that few red cells are produced This is termed erythroid hypoplasia and if no red cells are being produced erythroid aplasia Such a condition may be temporary or permanent A temporary depression of the marrow may be produced by transfusion

Infection may be the stimulus which causes an outpouring of granular leucocytes and such a marrow condition is termed myeloid hyperplasia A hypoplastic or aplastic appearance can occur with the myeloid series as it can with the megakaryocytes and such conditions can be specific although there is generally some overlap so that aplasia of one series is uncommon without some hypoplasia of other elements

Pathological stimuli whose nature is unknown may result in the marrow producing only one type of cell and one which may be alien to the marrow Thus in lymphatic leukaemia the marrow is producing only lymphocytes and in pernicious anaemia a megalo blastic hyperplasia results Such a condition may be termed a dysplasia

THE SPLEEN

The spleen is a soft organ with a capsule which penetrates its substance forming a framework of trabeculae The cut surface of the organ shows two types of pulp The red pulp is the material which can be scraped off with a knife blade and consists of all

CHAPTER 2

HAEMOGLOBIN

GENERAL

CHEMICAL STRUCTURE

HAEMOGLOBIN comprises most of the 35 per cent of solid materials in the red cell It is a chromoprotein that is a compound of a protein molecule with a non protein pigment (the prosthetic group) combined in a manner which confers characteristic properties on the conjugated protein The property possessed by haemoglobin is the ability to combine reversibly with oxygen to form oxyhaemoglobin without change in the valency of the iron in the prosthetic group

Haemoglobin has a molecular weight of 65 882 and is built up of 96 per cent globin and 4 per cent haem Globin is a simple protein composed of natural amino acids many of which are growth factors for various bacteria and is probably identical with serum albumen Haem is a ferro protoporphyrin complex an isomer of the porphyrin molecule which consists of four pyrrole rings linked by methene bridges

Theoretically fifteen such isomers are possible but only two occur in nature Porphyrins I and III are synthesized in the body glycine and acetic acid being essential in their production Each haem molecule contains one atom of Fe and haemoglobin consists of four such ferro protoporphyrin complexes united with globin Haemoglobin is a very soluble protein easily crystallizable and denatured by alkali (Fig 7)

DIETARY REQUIREMENTS

Protein is essential for the formation of haemoglobin but deficiencies do not necessarily result in anaemia neither does there appear to be any specificity of a particular amino acid when anaemia is induced by malnutrition Globin production makes preferential demands on the protein pool of the body so that dietary deficiency must be severe and sustained before anaemia develops Iron is essential for haemoglobin synthesis and copper and other trace elements may assist by acting as catalysts in the reactions

Dietary iron is ionized and put into solution in an acid medium by the hydrochloric acid of the gastric juice and kept in the ferrous

splenic reticulo endothelial system the haemoglobin being broken down to (*a*) an iron containing compound which is stored in the reticulo endothelial system for transmission to the marrow for re utilization and (*b*) bilirubin an iron free compound This is true bilirubin and gives an indirect van den Bergh reaction until it *has passed through the liver In the liver the bilirubin becomes* conjugated with glucuronic acid and gives a direct van den Bergh reaction The bilirubin glucuronide passes into the bile and some is oxidized to biliverdin In the gut it becomes sterco bilinogen or faecal urobilinogen and some is reabsorbed whilst the rest is passed as faecal urobilinogen or in oxidized form as urobilin The re absorbed substance passes into the blood stream and is excreted through the kidneys as urinary urobilinogen On exposure to light and air it becomes oxidized to urobilin

THE WHITE CELL

The life of the white cells is a matter of considerable argument Experiment suggests that most white blood cells exist in the circula tion for a few days only with the basophil having the longest life The white cells become senile in the blood stream appearing as smudge or smear cells basket cells and exploded cells The dis integrated bodies are removed by the phagocytic cells of the spleen

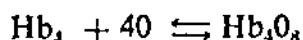
THE PLATELET

Platelets also *have a short life being removed from the circulation* within a matter of days Survival tests indicate a life of from 90 to 120 hours but this may be an underestimate

woman needs 15 mg of iron per day and a growing child the same, while a man requires 5 mg each day Deficiency of iron produces a characteristic anaemia

FUNCTIONS OF HAEMOGLOBIN

The vital function of haemoglobin is to carry oxygen although it also assists in buffering the *p*H of the blood and plays an important part in carbon dioxide transport It combines with oxygen in the proportion of one molecule of oxygen to one atom of iron the reaction being expressed in the equation



$$Hb_4 + 4O \leftrightarrows Hb_4O_8$$

a formula which allows of intermediate forms depending on the amount of oxygen available The oxygen combining power of blood in an adult male according to Haden is 20 9 volumes per cent which is equivalent to 15 6 g of haemoglobin per 100 ml of blood The Haldane standard is 14 77 g per cent, the oxygen combining power being 19 8 volumes per cent These are calculated on the basis that 1 0 g of haemoglobin can combine with 1 34 ml of oxygen a factor determined by Hufner using ox haemoglobin More recent work suggests that this figure should be amended to 1 36 ml Calculations which follow in this Chapter are based on the latter figure

NORMAL VALUES AND PHYSIOLOGICAL VARIATIONS

Females have a lower haemoglobin concentration than males the average being 13 7 g per cent The average for adult males is 15 6 g per cent while in newborn infants determinations on cord blood may reach 20 g per cent a figure which falls to 15 5 g after a few weeks At one year the average is 11 2 g and at 10 years 12 9 g per cent Although venous and capillary blood differ little in haemoglobin content there is some variation in determinations made from the same individual at different times Samples of blood taken at short time intervals differ significantly and it is reported that such variations are greater with blood from the ear than from the finger Haemoglobin levels fall between 6 a m and 10 p m and rise during sleep There is also a daily variation but such physiological variations would not appear to be of clinical significance

HAEMOGLOBIN PIGMENTS

Normally haemoglobin exists either combined with oxygen as oxyhaemoglobin or in its reduced form but blood also contains

state by the contents of the small intestine ascorbic acid assisting in this process by preventing oxidation The ferrous iron is absorbed in the duodenum and in the intestinal mucosal cells becomes ferric iron This is then linked with apoferritin an iron free protein to form ferritin which is in equilibrium with ferrous iron When plasma iron level falls ferrous iron passes into the blood stream is oxidized to ferric iron and is transported to the liver by an iron binding globulin called transferrin or siderophilin From the liver

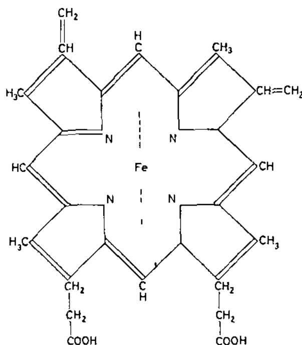


FIG 7—A suggested structure of haemoglobin

the ferritin is moved as required to the bone marrow This theory of selective absorption assumes that more ferrous iron can then be taken up by the cells of the intestinal mucosa and explains why under normal circumstances only the required amount of iron is absorbed

The normal daily loss of iron in desquamated skin cells sweat and urine has been estimated to average 1 mg Menstruation adds a loss of 14–28 mg each month and pregnancy demands 1–2 mg daily to meet the needs of the foetus Lactation entails a further loss for the nursing mother To counter these losses a normal

called Fraunhofer lines These correspond to elements in the sun and their position in the spectrum is constant

If a suitable thickness of haemoglobin solution is placed between the light and the slit black bands will be seen in the spectrum and these absorption bands are characteristic of the haemoglobin pigment concerned (Fig 9) Some direct vision spectroscopes are

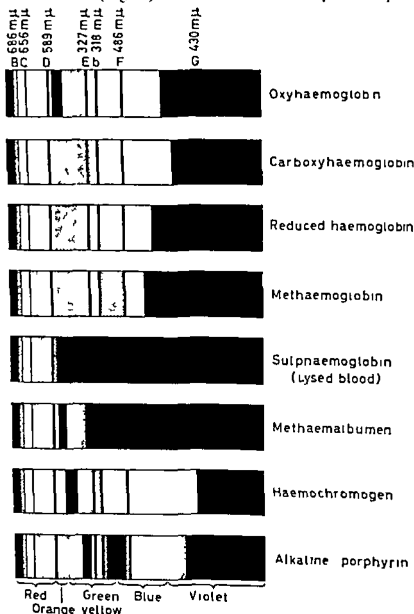


Fig 9 —Absorption spectra of the commoner haemoglobin pigments

small quantities of inactive pigments which are incapable of carrying oxygen The blood of a heavy smoker may contain 10 per cent of such pigments the main one being carboxyhaemoglobin Pathological amounts of methaemoglobin or sulphaemoglobin or both are found in cases of poisoning due to many drugs particularly those of coal tar origin Enterogenous cyanosis is a condition of uncertain aetiology characterized by abnormal amounts of intra corpuscular methaemoglobin The haemoglobin pigments active or not do not appear in the plasma in demonstrable quantities unless the red cells are being rapidly destroyed by a haemolytic process When haemolysis is taking place the pigments may be excreted in the urine if the renal threshold is exceeded

Small amounts of pigments derived from haemoglobin break down and by products of haemoglobin synthesis are found normally in small amounts in urine and faeces An increase in excreted urobilinogen may be indicative of excessive red cell destruction and an increase of coproporphyrins a sign of blood regeneration Abnormally large amounts of porphyrins are found in the urine in the condition known as porphyria This is an inborn error of metabolism which gives rise to many distressing symptoms and is often fatal The urine varies in colour from pink to almost black and contains large amounts of the pigments coproporphyrin I and uroporphyrin

USE OF THE SPECTROSCOPE

It is necessary in disorders involving haemoglobin pigments to identify and sometimes to measure the amounts of pigment in blood or urine This is done by means of the spectroscope an instrument which utilizes the fact that certain coloured substances absorb light The direct vision spectroscope (Fig 8) consists of a brass tube with an adjustable slit at one end At the other is an eyepiece and a lens system which can focus on the slit The tube holds either a diffraction grating or a series of three or more prisms alternately upside down and right side up of crown and flint glass If sunlight is allowed to fall on the slit the white light is split into its constituent colours and this solar spectrum is interrupted by a series of vertical lines

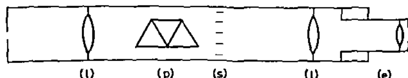


FIG 8 —The direct vision spectroscope (*l*) lens (*p*) prisms (*s*) scale (*e*) eye piece

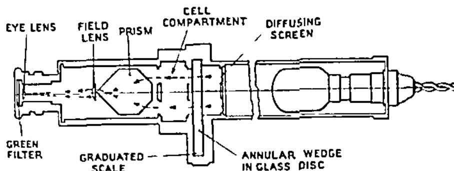


FIG 10—Optical system of the MRC photometer (*Reproduced by courtesy of C Davis Keeler, Ltd London*)

$$\frac{\text{Reading of test}}{\text{Reading of standard}} \times \text{strength of standard}$$

The galvanometer should give an infinity reading when there is no light transmission and a zero reading with 100 per cent transmission that is with a cuvette or fused glass cell containing only diluent To limit the light transmitted within a definite wavelength range a bright spectrum yellow green Ilford 625 filter is used A similar filter is used in the MRC photometer

Photo electric colorimeters are usually calibrated for use and, provided the solutions obey Beer s Law a straight line is obtained Such calibration curves should be made for every instrument since the machines vary individually Neutral grey screens of known optical density or grey solutions may be used as standards or as a means of checking the instrument Photo cells become tired with use and should then be changed If they become wet a reverse charge is obtained deflecting the needle nearer to zero rather than its resting point In this case also the photo cell must be replaced Should difficulty be experienced in obtaining full needle deflection the light source must be inspected since the bulbs become blackened with use and emit less light Photo electric colorimeters also employ a heat filter of heat resisting glass which absorbs the infra red portions of the spectrum which might have an effect on colour filters

The oxygen combining power of haemoglobin estimates only reduced haemoglobin and oxyhaemoglobin but by iron analysis the iron bound to inactive pigment is included The specific gravity method has an application in blood transfusion work in that it is a speedy method of deciding that the blood of a prospective donor is of a specific haemoglobin concentration

equipped with a wavelength scale but with others the absorption bands may be located in reference to the Fraunhofer lines

The reversion spectroscope gives two spectra of one solution one spectrum being above the other and reversed The apparatus may be used for the detection and even measurement of the amount of carboxyhaemoglobin in a sample of blood

THE ESTIMATION OF HAEMOGLOBIN

Haemoglobin may be estimated colorimetrically photometrically or by methods based on chemical or physical properties of the pigment A colorimetric method is one in which the haemoglobin pigment is brought to exact colour match with a known standard whilst a photometric method determines haemoglobin concentration by measuring colour intensity in terms of the light absorbing power of the solution at a specific region of the spectrum Chemical *methods involve either iron analysis or oxygen combining power* whilst with physical techniques the specific gravity of whole blood and plasma is estimated and haemoglobin values calculated

As oxyhaemoglobin solutions are difficult to match by eye the oxyhaemoglobin is usually converted to another compound for example carboxyhaemoglobin by an appropriate method and then diluted until it matches the standard The apparatus used consists merely of pipettes graduated to contain 0 02 ml and a rack for the test tube and standard The standard may be a sealed tube of the appropriate pigment or a permanent glass standard Some instruments utilize a double glass standard and a test tube square in section set between them to make comparison easier Care must be taken that the bore of the test tubes and sealed standard tubes is identical. This can be confirmed by matching the 20 and 100 marks on the two tubes

Photometric methods use either a visual instrument such as the MRC photometer (Fig 10) or photo-electric instruments With the MRC photometer haemoglobin is estimated as oxyhaemoglobin and its power of absorbing green light is compared with a neutral grey wedge The haemoglobin is read off directly as a percentage on the Haldane scale that is 100 per cent haemoglobin = 14 6 g per cent With photo-electric colorimeters a beam of light passes through the haemoglobin solution and the transmitted light falls on a photo-cell causing the generation of an electric current which is proportional to the intensity of the beam The strength of the current is measured by a galvanometer and haemoglobin concentration is calculated from

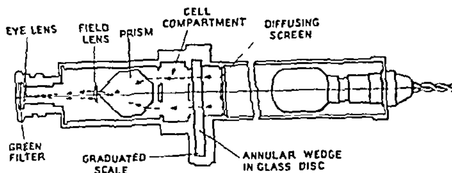


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TECHNIQUE

COLLECTION OF SAMPLES

Blood is usually collected either by skin prick or venepuncture

Skin pricks

Skin pricks are made with a cutting needle preferably in a region rich in capillary loops The skin at the base of the nail the ball of the finger or thumb and the ear lobe are suitable sites in the adult and the plantar surface of the heel in small infants The site selected must be neither cyanosed nor bloodless nor oedematous and should be briskly rubbed with alcohol to ensure that the capillary blood is not static in the vessels a condition which may produce anomalous results *The alcohol also sterilizes the skin The skin should be* puckered between finger and thumb and the puncture so made that when the skin fold is released the wound gapes and blood flows freely The wound must not be squeezed since this may result in dilution with tissue juices The first drop or two of blood is discarded, the blood then taken to the mark the outside of the pipette wiped clean and the sample delivered into the appropriate reagent It is advisable that a fresh sterile needle be used for each patient to avoid transmitting the virus of serum jaundice

Venepuncture

Venepuncture is usually made in one of the vessels in the antecubital fossa but other sites may be used in cases of difficulty Stasis must be avoided since it results in haemoconcentration and the puncture should be clean and blood withdrawn without undue suction The needle should be removed from the syringe and the blood delivered gently into anticoagulant Suitable anticoagulants are discussed in Chapter 3

COLORIMETRIC METHODS

Colorimetric methods of haemoglobin estimation are as follows

Carboxyhaemoglobin method

The procedure for the carboxyhaemoglobin method is as shown hereunder

(1) Place ammoniacal distilled water (0 4 per cent v/v) in the Haldane tube to the 20 mark and wash 0 02 ml of blood into it

(2) By means of a capillary pipette pass coal gas into the haemoglobin solution until it is cherry red in colour To prevent frothing first dip the tip of the pipette in caprylic alcohol
(3) Add the ammonia diluent drop by drop comparing with the standard until just under and then just over a perfect match Report the mean of the two readings

B S I specification for the colour standard defines 100 per cent as corresponding to 14 6 g per 100 ml by iron analysis and an oxygen capacity of 19 8 $\pm$ 0 2 volumes per cent of 14 77 g haemoglobin per cent

The method estimates reduced haemoglobin and oxyhaemoglobin and is quick and moderately accurate Sulphaemoglobin is not estimated but methaemoglobin can be included by first reducing with sodium hydrosulphite The method is subject to the errors of any continuous dilution technique and a supply of coal gas is essential Excess bilirubin introduces matching difficulties which can be avoided by first washing in saline the cells contained in 0 02 ml of blood, discarding the stained supernatant and then adding the requisite amount of ammonia diluent The standards tend to fade and should be checked at intervals of 6 months

Ammonia water is used as a diluent because slightly alkaline water prevents the turbidity which may occur due to precipitated globulins

Acid haematin method

The procedure for the acid haematin method is as shown here under

(1) Place the reagent (N/10 HCl) in the Sahli tube to the 20 mark and wash 0 02 ml of blood into it
(2) Allow the test to stand at room temperature for a period of time specified by the makers of the instrument and then dilute the brown solution drop by drop with N/10 HCl until the colour matches the glass standard The Sahli tube is graduated both in grammes per cent and haemoglobin percentages

The method measures only reduced haemoglobin and oxyhaemo globin and has another and more serious disadvantage in that the colour takes a variable time to develop In adults much of the colour develops in 10 minutes but in infants the process may take considerably longer due to the resistance of foetal haemoglobin to acid treatment The standard of 17 3 g per 100 ml is no longer used most instruments being standardized to 14 8 g per 100 ml The brown-glass standards may fade and the acid haematin being present in

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The haemoglobin values may be estimated by using standards either of grey screens of known optical density, or Thompson's inorganic grey solution which, diluted with half its volume of distilled water has an optical density of 0 475

Thompson's solution

Chrome alum $Cr_2(SO_4)_3 K_2SO_4 24H_2O$	16 67 g
Copper sulphate $CuSO_4 5H_2O$	33 33 g
Cobalt ammonium sulphate $CoSO_4(NH_4)_2SO_4 6H_2O$	39 50 g
Potassium dichromate $K_2Cr_2O_7$	1 12 g

Dissolve in 1 litre of distilled water at room temperature and store in a glass stoppered bottle for 6 weeks during which time the colour changes from pink to grey due to a decrease in the hydration of the chromium ions It should have a *p*H of 12 64

The oxyhaemoglobin method using the *MRC photometer* with Ilford 625 filter is as follows Wash 0 02 ml of blood into 2 0 ml ammoniated water make up to 4 0 ml and place in the special cuvette Place the appropriate filter in position over the eye piece and rotate the grey wedge by the knurled nut at the side of the instrument until the light transmitted by the test solution matches that in the adjacent half field which is transmitted by the grey wedge through a blank of ammonia water The haemoglobin value is read directly as a percentage on the Haldane scale The instrument may be checked with a neutral grey screen Care must be taken that all glass surfaces are clean since dirt may affect the light fields un equally The method is subject to a small error at all points but is still suitable as a routine technique

Alkaline haematin method

The procedure for the alkaline haematin method is as shown hereunder

Gibson Harrison standard

Chromium potassium sulphate $CrK(SO_4)12H_2O$ Analar	11 61 g
Anhydrous cobaltous sulphate $CoSO_4$	13 1 g
Potassium dichromate $K_2Cr_2O_7$	0 69 g

The chemicals are dissolved in 500 ml of distilled water 1 8 ml N H SO_4 added heated to boiling for 1 minute cooled and the volume made up to 1 litre with distilled water The ready made standard may be purchased of British Drug Houses and is equivalent

colloidal form may be turbid unless mixed quickly with the acid These disadvantages may be set against the simplicity of the method, and ready availability of the reagent but it is not recommended as a routine method although it is extensively used in Continental countries

Standardization of a photo electric colorimeter for the oxyhaemoglobin method

A suitable photo electric colorimeter for the oxyhaemoglobin method is that supplied by Gallenkamp, London (catalogue number b-3615) It is a self contained unit equipped with a sensitive micro ammeter calibrated with an inverse logarithmic scale A sliding carrier which can hold two cells moves between stops so that successive readings may be rapidly taken The instrument is fitted internally with a constant voltage transformer which gives very steady results when connected to a frequency controlled mains supply At the top of the instrument is a brief instruction plate showing the fundamental operating sequence

To standardize the instrument adjust the galvanometer needle to infinity and using an Ilford filter (bright spectrum yellow green number 625) adjust the light to give a zero reading with a blank of 4 0 ml ammoniated water in a glass cell A second cell should also give a zero reading with this solution Well mix blood of known haemoglobin content and wash 1 0 ml into 200 ml of ammoniated water From this solution make dilutions from 10 to 90 per cent, and in the second glass cell determine the extinction values of these and the original—ensuring that a zero reading is obtained with the blank each time Construct a calibration curve by plotting the extinction values against haemoglobin percentages The graph may then be used to construct a table of all significant galvanometer readings correlated with haemoglobin concentrations in grammes per 100 ml of blood and percentages on the Haldane scale (100 per cent–14 6 g per cent)

The oxyhaemoglobin method procedures

The oxyhaemoglobin method using the *photo electric colorimeter* is as follows Wash 0 02 ml of blood into 4 0 ml ammoniated water and determine the extinction value in the colorimeter Read the haemoglobin value from the table (*see* above) Should the extinction value be above 0 7 the blood sample should be diluted to ensure that the reading is made in the accurate part of the scale The method is quick and accurate and is very suitable for routine estimation It estimates oxyhaemoglobin and reduced haemoglobin

is blood of known haemoglobin content diluted as above in Drabkin solution It is usual to have standards of different strengths but the solutions of cyanmethaemoglobin are stable for years and so may be regarded as permanent The method estimates oxyhaemoglobin reduced haemoglobin methaemoglobin and carb oxyhaemoglobin all the pigments being converted to cyan methaemoglobin

CHEMICAL METHODS OF HAEMOGLOBIN ESTIMATION

Iron analysis

In iron analysis wash 2 5 ml of oxalated blood with glass distilled water from a pipette graduated ' to contain into a 50 ml Pyrex beaker Add a few drops of iron free concentrated nitric acid Cap the beaker with filter paper and slowly evaporate the mixture to dryness Heating should be gradual to avoid spattering Then heat more strongly until fuming ceases and ash overnight in a muffle furnace at 450° C Dissolve the ferric ash by warming with 2 ml of iron free concentrated hydrochloric acid and dilute to about 20 ml with glass-distilled water Add 1 ml of 20 per cent (n/1) potassium thiocyanate as an indicator and titrate the mixture to a colourless end point with undiluted (15 per cent) titanous chloride The reagent is added from a micrometer syringe burette (Burroughs Wellcome London) which has a capillary outlet with a turned up tip dipping into the iron solution Stir the mixture con tinuously by means of CO_2 which passes into it from a narrow tube Using the same pipette which was used for the blood wash 2 5 ml of iron standard into a similar beaker and titrate Haemoglobin is calculated from the blood iron in the following manner

$$\text{Blood iron (mg per cent)} = \frac{\text{Titration of test}}{\text{Titration of standard}} \times 50$$

$$\text{Haemoglobin (g per cent)} = \frac{\text{mg Fe per cent}}{3\ 38}$$

Iron standard

The procedure for the iron standard method is as follows

Dissolve ferric ammonium sulphate 43 18 g in water with 100 ml concentrated hydrochloric acid and make up to 1 litre This stock solution contains 5 mg Fe per ml The working standard is a 1 in 10 dilution of this standardized by $TiCl_3$ titration against a solution of pure iron wire which is ashed in exactly the same manner as blood

The technique is obviously not suitable as a routine method but is very accurate for research and standardization procedures It

to 16 0 g haemoglobin per 100 ml of blood when used in this technique

Well mix 0 05 ml of blood with 4 95 ml of N/10 NaOH and heat in a boiling water bath for exactly 4 minutes Boil a sample of the standard at the same time Cool quickly and match both in the photo electric colorimeter using the Ilford 625 filter A fresh sample of standard is heated for each test or batch of tests

Total haemoglobin is measured by this technique which is simple and accurate Hyperglycaemic or lipaemic blood may cause tur bidity which can be dispersed by the addition of 0 1 ml of Teepol to 10 ml of the alkaline haematin solution The artificial standard of Gibson and Harrison must be boiled, since only after heating—which alters the ionization of the salts—does its power to absorb green light approximate to that of alkaline haematin

Acid alkali method

The procedure for the acid alkali method is as shown hereunder

Well mix 0 05 ml of blood with 4 0 ml N/10 HCl and allow to stand 20–30 minutes Then add 0 95 ml of N NaOH and invert the tube several times After standing for at least 2 minutes match the test in the photo electric colorimeter against a grey screen or grey solution which has been calibrated for this technique The usual Ilford 625 filter is used

The only advantage of the method is that heating is unnecessary but although total haemoglobin is estimated the technique is time consuming

Cyanmethaemoglobin method

The procedure for the cyanmethaemoglobin method is as shown hereunder

Drabkin solution

Sodium bicarbonate $NaHCO_3$	1 0 g
Potassium cyanide KCN	0 2 g
Potassium ferricyanide $K_3Fe(CN)_6$	0 2 g

Dissolve the chemicals in 1 litre of distilled water

Add 0 02 ml of blood to 5 0 ml of Drabkin solution stopper the tube with a rubber bung and invert several times After the test has stood at room temperature for at least 10 minutes match in the photo electric colorimeter against the standard The standard

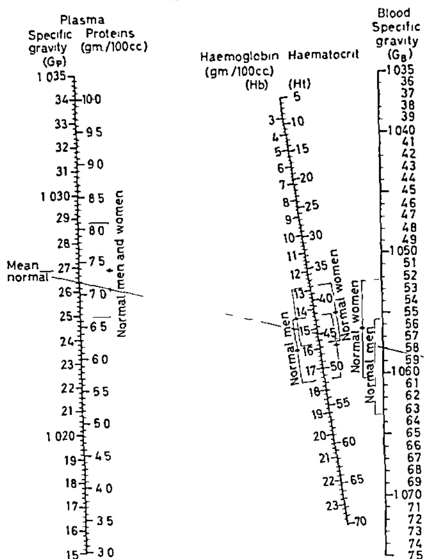


FIG 11 —Line chart for calculating plasma proteins haemoglobin and haematocrit from gravities of plasma and blood Method 1 Read off plasma proteins from the two scales on line to left 2 Read off haemoglobin on haematocrit by stretching thread across G_B and G_p numbers on outside lines The result is found on inner line 3 Read off approximate haemoglobin using whole blood only by stretching thread across G_B and mean normal plasma proteins (6 9 g per cent) Note Plasma scales read up others down (*Reproduced by courtesy of Donald D Van Slyke Brookhaven National Laboratory Upton New York*)

Preparation of copper sulphate solutions

In the preparation of copper sulphate solutions the procedure is as follows

must be remembered that iron bound to inactive pigments is included in the estimation hence the results will not agree completely with those obtained by the oxygen capacity method

Oxygen capacity method

A detailed description of the oxygen capacity method and the correction tables are beyond the scope of this book and the interested reader is referred to the Peters and Van Slyke work *Quantitative Clinical Chemistry* Volume II In brief blood is aerated in a separation funnel and introduced into the Van Slyke apparatus where it reacts with a laking agent and potassium ferricyanide The mixture is shaken until the volume of evolved gases (O N and CO_2) is constant and the CO_2 removed with NaOH Temperature barometric pressure and tension of aqueous vapour are included with a correction factor for nitrogen and physically dissolved gases in the calculation Each ml of oxygen capacity represents 0 735 g of haemoglobin

PHYSICAL METHOD OF ESTIMATING HAEMOGLOBIN

Copper sulphate specific gravity method procedure

The copper sulphate specific gravity method of estimating haemoglobin is as follows

Collect venous blood into heparin (0 1–0 2 mg per ml) and allow a drop of well mixed blood to fall from a height of 1 cm into a series of solutions of copper sulphate Heparinized blood does not keep well for this test and must be used within a few hours The drop of blood becomes coated with copper proteinate and having penetrated 2–3 cm into the liquid either remains stationary or begins to rise or fall For a period of 15 seconds the behaviour of the drop is observed If it remains stationary it is of the same specific gravity as the solution if it falls it is heavier and if it rises it is lighter All drops eventually fall so that the fluids automatically clean themselves The specific gravity of both whole blood and plasma are determined in this manner and the haemoglobin concentration read from the line chart (Fig 11)

The technique is easy and is more accurate than methods involving colour matching by eye except with abnormal bloods as for example hyperglobulinaemia An approximation of haemoglobin may be made using whole blood alone and since no gas or electricity is needed the method is ideal for field work The plasma proteins and haematocrit is not reliable The standards must be discarded after they have been used once for every ml of solution

TABLE II
STANDARD SOLUTIONS

Standard solutions for plasma		*Standard solutions for whole blood*			
S G plasma	*Volume of stock solution per 100 millilitres standard*	*S G blood*	*Volume of stock solution per 100 millilitres standard*	*S G blood*	*Volume of stock solution per 100 millilitres standard*
	Millilitres		*Millilitres*		*Millilitres*
1 015	13 9	1 035	34 3	1 055	54 3
16	14 9	36	35 3	56	55 3
17	15 85	37	36 3	57	56 3
18	16 8	38	37 25	58	57 3
19	17 8	39	38 2	59	58 3
20	18 8	40	39 2	60	59 3
21	19 8	41	40 2	61	60 3
22	20 75	42	41 2	62	61 3
23	21 7	43	42 2	63	62 3
24	22 7	44	43 2	64	63 35
25	23 7	45	44 2	65	64 4
26	24 7	46	45 2	66	65 4
27	25 7	47	46 2	67	66 4
28	26 65	48	47 2	68	67 4
29	27 6	49	48 2	69	68 4
30	28 6	50	49 2	70	69 4
31	29 6	51	50 2	71	70 4
32	30 6	52	51 25	72	71 45
33	31 6	53	52 25	73	72 5
34	32 6	54	53 3	74	73 5
35	33 6	55	54 3	75	74 5
36	34 6				

SPECTROSCOPIC EXAMINATIONS

In spectroscopic examinations the procedure is as shown here under

Adjust the slit of the spectroscope and focus the eye piece until a clear spectrum is seen and the Fraunhofer lines are sharply defined Horizontal lines may sometimes be seen and are due to specks of dirt on the slit which may be cleaned with a camel hair brush The pigment solution must be clear and appropriately diluted The importance of this latter may be demonstrated by examining a strong solution of oxyhaemoglobin A single broad band is seen but on dilution separates into two bands typical of the oxyhaemo

Place 510 0 g fine crystals of copper sulphate ($CuSO_4 5H_2O$) A R in a 4 litre bottle Fill a 1 litre volumetric flask to the mark with distilled water take the temperature of the water and add from a burette enough additional water to the flask to bring the volume up to that indicated in Table I for addition to 170 0 g of sulphate Empty the water from the flask into the bottle containing the sul phate and let the water from the flask drain into the bottle for 2 minutes Repeat the process three times

To prepare each standard measure the volume of stock solution indicated in Table II into a 100 ml volumetric flask Add water at the same temperature in a rapid stream until the surface approaches the neck of the flask rotating the flask all the time Continue adding water without rotating until the 100 ml mark is reached and invert the flask to complete mixing Decant the solution into a labelled bottle and rinse the flask with distilled water before using the next standard

TABLE I

VOLUMES OF WATER TO ADD TO 170 GRAMMES OF $CuSO_4 5H_2O$ TO PREPARE STOCK SOLUTION OF COPPER SULPHATE[1] S G 1 100

Temperature of water	*Millilitres of water to 170 grammes $CuSO_4 5H_2O$*
C	
10	1003 6
12	1003 8
14	1004 0
16	1004 3
18	1004 7
20	1005 1
22	1005 5
24	1006 0
26	1006 5
28	1007 0
30	1007 7
32	1008 3
34	1008 9
36	1009 6
38	1010 4
40	1011 2

[1] The stock solution contains 1002 5 g of water per 170 g of $CuSO_4 5H_2O$ The volumes of water given in the Table are 0 8 ml more than theoretical in order to allow for adherence of this amount to the inside of the flask after 2 minutes drainage

Methaemalbumen—Dissolve haemin hydrochloride in N/100 NaOH to a concentration of 1 mg per ml Add one volume of this alkaline haematin to five volumes of serum or plasma A clear brown solution of methaemalbumen is immediately produced showing a well defined band in the red portion of the spectrum In pathological specimens the absorption band may be very faint but the presence of methaemalbumen is confirmed by Schumm s test Cover the test plasma with ether and add one tenth of its volume of ammonium sulphide Shake the tube to mix the reagents and examine with the spectroscope An ammonium haemachro mogen is formed from methaemalbumen, this is shown by the disappearance of the band in the red and the appearance of a sharply defined band in the green

globin Hold the tube containing the solution against the slit and bring upwards so that the empty part of the tube is observed first For practice and demonstration purposes some of the haemoglobin pigments are easily prepared from defibrinated or oxalated blood It may be necessary to centrifuge the preparation to obtain clear samples for spectroscopy

Oxyhaemoglobin—Lyse aerated blood with distilled water and further dilute until two bands are seen a narrow one near the D line and a broader in the green near the E line

Haemoglobin (reduced haemoglobin)—Add a small quantity of solid sodium hydrosulphite to a preparation of oxyhaemoglobin showing two well defined bands The blood changes in colour from red to violet and the two bands of oxyhaemoglobin are replaced with a single broad band between D and E

Carboxyhaemoglobin—By means of a capillary pipette pass coal gas into a solution of oxyhaemoglobin in ammoniated water until it is cherry red in colour The two bands seen differ from oxyhaemoglobin only in that they are slightly nearer the violet end of the spectrum but the addition of sodium hydrosulphite fails to reduce the carboxyhaemoglobin Spectroscopically small amounts of carboxyhaemoglobin are not detected chemical methods being more sensitive

Methaemoglobin (neutral methaemoglobin)—Add a few drops of freshly prepared 10 per cent potassium ferricyanide to an oxyhaemoglobin preparation and shake The blood becomes brownish in colour and on dilution a single very dark band is seen to the left of the D line Other faint bands between D and E and another nearer F may be seen if the blood is sufficiently diluted Keeping the preparation before the spectroscope add a little solid sodium hydrosulphite and the spectrum will be seen to change through that of oxyhaemoglobin to reduced haemoglobin

Sulphaemoglobin—Add 0 1 ml of 0 1 per cent aqueous phenyl hydrazine hydrochloride and one drop of water saturated with H_2S to 10 ml of an oxyhaemoglobin solution The preparation will not keep and should be prepared just before use To differentiate the spectra of sulphaemoglobin and methaemoglobin add sodium hydrosulphite The band in the red disappears if methaemoglobin is present but remains unchanged if sulphaemoglobin is there The spectra are also altered by the addition of 1 per cent potassium cyanide which produces cyanhaemoglobin with a spectrum closely resembling reduced haemoglobin The change takes place within a few minutes in the case of methaemoglobin but sulphaemoglobin may take as long as 24 hours

Heparin

Heparin is a physiological anticoagulant produced by the liver and used in a concentration of 0 1–0 2 mg per ml of blood It is expensive causes leucocytes to clump and requires very thorough mixing with the blood Red cell size and shape are unaltered making it valuable as a standard when testing new anticoagulants

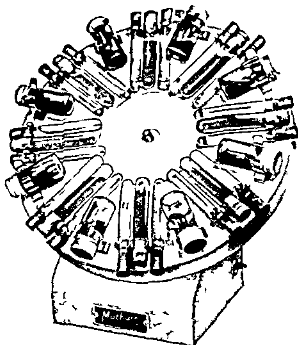


FIG 12—Suspension mixer (Matburn) the clips on the rotating table can be altered to suit many sizes of tubes and vials (*Reproduced by courtesy of Matburn Ltd London*)

NORMAL VALUES AND PHYSIOLOGICAL VARIATIONS

Red cell count

The normal red cell count in man is 4 5–6 5 millions per c mm in women 3 9–5 6 millions and in cord blood of infants 4–6 millions per c mm Peripheral blood in newborn infants often has a higher red cell count than cord blood but the numbers fall as the child grows to rise again when mixed feeding provides the necessary nutrients Adult levels are reached at or about puberty and until this age male and female children differ very little It is probable that the normal female red cell count is related to menstrual loss Muscular activity and emotional disturbance cause a temporary

CHAPTER 3

PRACTICAL BLOOD COUNTING

APPARATUS AND SOLUTIONS DATA

COLLECTION OF SAMPLES

BLOOD may be collected in haemocytometer pipettes in which dilution is made in the bulb or the sample may be delivered into a measured volume of diluting fluid in a tube Both methods are subject to inaccuracies but the second is considerably cheaper since blood pipettes are both fragile and expensive Venous blood collected into an anticoagulant must be thoroughly mixed before sampling preferably in a suspension mixer of the Matburn type (Fig 12)

ANTICOAGULANTS

Three anticoagulants only are permissible for cell counting they are given below in order of superiority

Sequestrene

Ethylenediamine tetra acetic acid (EDTA) (Sequestrene) is a non toxic chelating agent used in the treatment of lead poisoning The di potassium salt is the anticoagulant of choice since it is readily soluble prevents the clumping of platelets and preserves the cell morphology for a considerable time so that accurate counts may be performed on blood kept overnight It is used in a concentration of 1–2 mg per ml of blood The di sodium salt has identical properties but is much less soluble

Heller and Paul mixture

Dry ammonium oxalate causes red cells to swell and potassium oxalate causes them to shrink but the mixture recommended by Heller and Paul maintains red cell size and shape for some hours However the leucocytic nuclei quickly undergo extensive change platelets are clumped and red cells slowly become crenated The salts which act by precipitating calcium as insoluble oxalate are used in the proportion of 6 mg ammonium oxalate and 4 mg potassium oxalate to 5 ml of blood

engraved with rulings and are sometimes coated with a metal to give a bright line effect After use counting chambers are washed under running water, dried with a lint free cloth and stored dry B S I specifications exist for both counting chambers and coverslips The rulings of more commonly used chambers are illustrated (Fig 13) together with the Manner s chamber which was developed for eosinophil counts and is most convenient since the volume enclosed by the entire ruling is 10 c mm Inequality in cell distribution is very obvious in this chamber longitudinally

Pipettes

Haemocytometer pipettes are cleaned by a vacuum pump using first water then acetone Blocked pipettes are cleared with a bristle, never a wire Should the tip be blocked it can be cleared without damage by dabbing with a nail brush while the pipette is still on the vacuum pump Small dried clots should never occur with proper use but should they do so may be cleared with 40 per cent caustic soda Pipettes with chipped tips must be discarded B S I specifi cations exist for haemocytometer pipettes

DILUTING FLUIDS

Ideally red cell fluids should preserve the red cells without clump ing and white cell fluids should destroy the red cells while preserving and staining the white cells Eosinophil fluids should destroy red cells and leucocytes other than eosinophils which should be stained Platelet fluids should not clump the platelets and preferably should stain them but this latter refinement is unnecessary when using phase contrast microscopy Fluids generally should be of such a specific gravity that cells do not sediment before the chamber can be filled

Modified Rees Ecker solution

The modified Rees Ecker solution comprises 1 per cent formalin in 3 per cent sodium citrate This simple fluid satisfies the criteria of a good red cell fluid and with the addition of 1 0 ml 0 5 per cent saline solution of brilliant cresyl blue to every 19 ml of diluent, makes an ideal platelet fluid The mixture is filtered after the addition of the dyestuff

Hayem s solution

Hayem s solution comprises

Sodium chloride	0 5 g	Sodium sulphate	2 5 g
Mercuric chloride	0 25 g	Distilled water	100 ml

increase in the red-cell count, and the mountain dweller exposed to rarefied atmospheres has a higher count than the inhabitant of low lying areas

White cell count

The normal white cell count has a range of 4 000 to 10 000 per c mm but the upper or lower limits may be quite abnormal for a particular individual The normal values for each type of leucocyte also vary widely neutrophils ranging from 40 to 70 per cent of the total lymphocytes 20–50 per cent monocytes 2–10 per cent eosinophils 1–6 per cent and basophils 0–1 per cent There is no sex difference in total or differential leucocyte counts The newborn infant has a white cell count of 10 000 to 25 000 per c mm most of the cells being neutrophils but within a few days the count falls to 6 000–10 000 per c mm the adult ratio of neutrophils to lymphocytes being reversed At about puberty adult numbers and proportions obtain but during childhood the white cell count fluctuates widely for no apparent reason

Leucocyte count

Total leucocytes vary in number from day to day and during the day Capillary blood may show higher counts than venous blood particularly where there is stasis Exercise results in a neutrophilia while exhaustion produces the opposite effect Over exposure to solar radiation may produce a lymphocytosis Eosinophils are increased in allergic conditions and decreased by the action of ACTH

Platelet count

The platelets normally vary in numbers from 150 000 to 400 000 per c mm the method used being important as far as the normal value is concerned There is a daily fluctuation of as much as 6–10 per cent and newborn infants have lower counts than adults Prior to menstruation there is a decrease in platelet numbers The count on capillary blood is usually lower than that on venous blood due to the clumping of platelets on the edges of the skin wound

APPARATUS

Counting chambers are heavy glass slides in the centre of which are single or double platforms The platforms are separated from one another and from the rest of the chamber by grooves or moats Two transverse bars are so ground that a coverslip placed on them is exactly 0 1 mm or 0 2 mm above the platforms this depth varying according to the type of chamber The platforms are

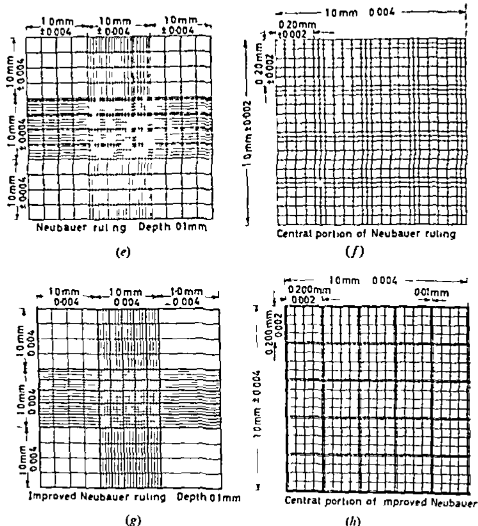


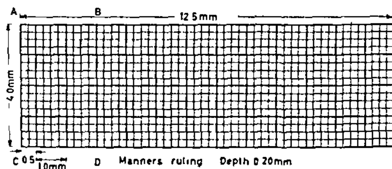
FIG 13 —Rulings of the commonly used counting chamber

This is not a satisfactory red cell fluid The mercury causes precipitation of globulins with marked rouleaux formation in cases of *hyperglobulinaemia* *Actual clumping* occurs when cold agglu tinins are present in the blood

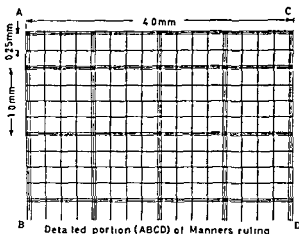
Toison s solution

Toison s solution is

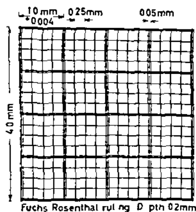
Sodium chloride	1 g	Methyl violet 6B	0 025 g
Sodium sulphate	8 g	Glycerine	30 *ml*
Distilled water	180 ml		


A
B
12 5 mm
40 mm
C 0 5
10 mm
D Manners ruling Depth 0 20mm

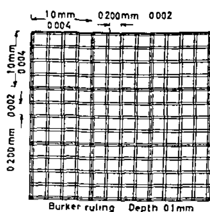
(a)


A
C
4 0 mm
0 25 mm
1 0 mm
B
D
Deta led portion (ABCD) of Manners ruling

(b)


1 0 mm
0 004
0 25 mm
0 05 mm
4 0 mm
Fuchs Rosenthal rul ng D pth 0 2mm

(c)


1 0 mm
0 004
0 200 mm
0 002
1 0 mm
0 004
0 002
0 200 mm
Burker ruling Depth 0 1 mm

(d)

ERRORS IN BLOOD COUNTING

The errors possible in blood counting may be classified as those of a personal technical and statistical nature Personal bias may cause a technician to consistently obtain higher or lower counts than the majority of other workers and although training will minimize *this, it will still be obvious on comparison* Technical errors include those arising in the collection of the sample, they are collecting from a cyanosed bloodless or oedematous area squeezing the skin puncture or haemoconcentration due to stasis arising from prolonged application of a tourniquet Included in this category must also be sampling from an inadequately mixed venous sample Pipetting errors arise from over or underdilution dirty chipped or badly calibrated pipettes and inadequately mixed suspensions Chamber errors include over or underfilling the chamber, counting before the cells have settled or allowing the edges of the preparation to dry before counting Counting the cells in a chamber containing air bubbles or dirt introduces errors since the bubbles exert a force in all directions pushing the cells away while dirt attracts cells particularly leucocytes

Using perfect apparatus and under ideal conditions the chamber still shows an error which is inherent in the distribution of the cells This irregular or random distribution of cells has certain characteristics by which it is possible to state that the number of cells in one area will differ from the average within a definite range The error depends on the fact that the random distribution follows Poisson s Law and can be calculated since it is proportional to the square root of the number of cells counted The measure of variation used is standard deviation or sigma (σ) and is expressed as $\sigma = \sqrt{M}$ where M is the average number of cells in the area under investigation In effect this means that with a true red cell count of 5 0 millions per c mm using a dilution of 1–200 the average number of cells counted in 80 small squares of the Neubauer chamber would be 500 and the *standard deviation* $\sqrt{500} = 22$ *(approximately)* *In* 95 per cent of similar areas counted the range would be 500 ± 20 or 456–544 The other 5 per cent would be outside this range If the coefficient of variation is calculated $\left(V = \frac{\sigma}{M}\right)$ the figure of 4 4 per cent is obtained and it will be seen that the error ($\pm$ 2V = 8 8 per cent) is quite considerable Since the percentage field error varies inversely as standard deviation however the more cells that are counted the less the error The field error is inherent in the cell distribution and is common to red-cell counts white cell counts and platelet counts

This is a satisfactory diluting fluid for both red and white cells in many respects but fungi and yeasts grow very readily in this medium

Turck s solution

Turck s solution comprises 2 per cent glacial acetic acid coloured with methyl violet this is a satisfactory white cell diluting fluid

Delaney s solution

Delaney s solution is N/10 HCl coloured with safranin and is a satisfactory white cell diluting fluid

Randolph s stain

Randolph s stain comprises

Solution A	0 1 per cent methylene blue propylene glycol	50 ml
	Distilled water	50 ml
Solution B	0 1 per cent phyloxine in propylene glycol	50 ml
	Distilled water	50 ml

Equal parts of solutions A and B are mixed freshly every day and filtered This stain is useful for both total white cell and eosinophil counts

Pilot s stain

Pilot s stain comprises

Propylene glycol	50 ml
Distilled water	40 ml
1 0 per cent phyloxine in water	10 ml
1 0 per cent sodium carbonate in water	1 0 ml

After filtration the stain is stored at room temperature It is excellent for eosinophil counts

Lempert (Kristensen s) solutions

Lempert s solutions are as follows

Solution A	Sodium citrate	1 0 g	The reagents are dissolved in distilled water at 45 C
	Mercuric chloride	0 002 g	
	Brilliant cresyl blue	0 2 g	

Solution B Freshly prepared 20 per cent urea in water Equal volumes of A and B are mixed when required and filtered Batches of urea vary in their power of erythrolysis so that the strength of the solution may need to be raised

This is a standard fluid for platelets but the method has no advantage over the use of Rees Ecker fluid plus brilliant cresyl blue

TOTAL LEUCOCYTE COUNT

The procedure in total leucocyte counting is as follows

(1) Take blood to 0 5 mark of the white-cell pipette wipe the outside of the pipette and take diluting fluid to the 11 mark and shake thoroughly

or

Make a 1 200 dilution of blood by adding 0 05 ml of blood to 0 95 ml of diluent

(2) Prepare the counting chamber and fill by capillary attraction [1]

(3) Count the cells in as many 1 mm × 1 mm squares as may be necessary to give a total of at least 100 cells

Calculations are easily made from such counting areas

ELECTRONIC COUNTING OF BLOOD CELLS

Several types of machines exist for automatic blood cell counting and that supplied by Casella (Electronics) Ltd is taken as an example The dark ground images of the cells are projected on to a screen having a slit in its centre behind which is a photomultiplier The pulses from the multiplier are amplified and counted The area scanned is 10 sq mm and time taken for the count no more than one minute The machine may be used for both red cell and white cell counts and results are reproducible to within ± 1 per cent from the same filling The chamber is of the same type as for visual counts but has no rulings Using a dilution of 1 1 000 the chamber is filled and placed in position on the stage of the focus checked The starter switch is operated and the machine does the rest For red-cell counts the number on the counter is multiplied by 1 000 and a correction applied from the coincidence correction scale For white cell counts dilute 1 100 and multiply result by one hundred subtracting the figures on the correction scale

TOTAL EOSINOPHIL COUNT

As a test of function of adrenal cortex the total eosinophil count is done before and 4 hours after administration of ACTH

The procedure is as follows

(1) Fill leucocyte pipette to 1 0 mark with blood wipe the outside of the pipette and take the fluid of Randolph or Pilot to the 11 mark

(2) Shake the pipette and allow to stand 20 minutes before filling the chamber

[1] Using haemocytometer pipettes ensure that one third of the pipette contents is discharged before filling the chamber

[2] Alternatively make a 1 10 dilution of a bulk method

Another error of distribution is that which arises in the filling of the chamber for leucocyte counts The density of leucocytes in the counting chamber increases progressively from the edge of the coverslip where filling commenced The average value is reached at the centre of the flooded area and the longer the filled area the less is the rate of change This means that to increase the accuracy of white cell counts the coverslip must be so placed that the ruled area is exactly central and preferably a single chamber used

TECHNIQUE

RED CELL COUNT

Red-cell counting procedure is as follows

(1) Take blood to the 1 0 mark of the red-cell pipette wipe the outside and take diluting fluid to the 101 mark and shake thoroughly

or

Make a 1 200 dilution of blood by adding 5 c mm of blood to 995 c mm of fluid using Strong s pipettes

or

Make a 1 200 dilution of blood by adding 20 c mm of blood to 4 ml of diluting fluid

(2) Set the coverslip squarely over the ruling so that the diffraction rings are seen where the coverslip is opposed to the supporting bars

(3) Shake the blood mixtures and allow the chamber to fill by capillary attraction[1] from a haemocytometer or capillary pipette with the chamber held at an angle and allow 3 minutes for the cells to settle

(4) Using the Burker chamber count the cells in three of the 3 mm × 0 05 mm rectangles including the cells touching the top and right hand margins Since the chamber is 0 1 mm deep the volume under examination is 0 045 c mm and with a 1 200 dilution the number of red cells in millions per c mm is given by

$N \times \frac{1}{0\ 045} \times 200 = N \times 4\ 440$ where N is the number of cells counted

Using the Neubauer chamber count the cells in 80 of the smallest squares that is one fifth of the central ruled area counting those cells which touch the top and right hand margins of each block of 16 squares The volume under examination is 0 02 c mm and with a 1 200 dilution the number of red cells per c mm is given by $N \times 50 \times 200 = N \times 10\ 000$ where N is the number of cells counted

Note The area counted should be adjusted for anaemic bloods so that at least 1 000 cells are counted

[1] Using haemocytometer pipettes ensure that one third of the pipette contents is discharged before filling the chamber

which consists of a capillary tube graduated in thousandths of a millilitre and having a capacity of 0 03 ml and a bulb of 5 ml capacity

(1) Venous blood is collected and 4 ml added to 6 ml of 1 3 per cent sodium oxalate and the mixture placed in the sedimentation chamber for 3½ hours
(2) 5 ml of the supernatant (equivalent to 1 ml of blood) together with a trace of red cells is removed and loaded in the thrombocytocrit
(3) The filled thrombocytocrit is centrifuged at 3 500 r p m for 1½ hours and the white layer of platelets in the capillary portion read in thousandths of a millilitre
(4) The percentage of platelets in whole blood is obtained by moving the decimal point two places to the right Normal range 0 35–0 67 per cent

(3) Using Manner s chamber count the entire ruled area No calculation is necessary Using the Fuchs Rosenthal chamber count the entire ruled area in four chambers and divide the total by 1 28

Note If the illumination is intense the eosinophils stand out brilliantly as the only stained cells

PLATELET COUNT

Platelet counting techniques are as follows

Dacie technique

(1) Venous blood is collected into Sequestrene and diluted 1 100 with modified Rees Ecker solution tinted with brilliant cresyl blue
(2) The diluted blood is thoroughly mixed the counting chamber filled and placed in a moist chamber for 20 minutes to allow the platelets to settle
(3) The platelets appear as refractile blue bodies and the number in 1 sq mm is counted

Lempert Kristensen technique

(1) Take the mixed stain in a leucocyte pipette to the 0 5 mark prick the ear and take blood to the 1 0 mark Quickly follow with stain to the 11 mark
(2) Shake the pipette for one minute
(3) After a few minutes shake the pipette again and fill the chamber
(4) Leave the filled chamber 30–60 minutes in a moist chamber to allow red cells to lyse and platelets to settle
(5) Count the platelets in 1 sq mm

The number per c mm is given by N × 200 where N is the number of platelets counted

Ammonium oxalate method

(1) Take 1 per cent ammonium oxalate to 0 5 mark in leucocyte pipette
(2) Take blood from freely flowing skin prick to 0 5 mark and complete filling of pipette with ammonium oxalate solution
(3) Shake pipette fill counting chamber and allow to stand 10 minutes in moist chamber
(4) Count platelets in centre ruled area preferably by phase contrast Calculation as for white count

Note Filter ammonium oxalate solution before use

Platelet counting by volume (Van Allen)

This method is a substitute for counting It requires a spherical sedimentation chamber of 20 ml capacity and a thrombocytocrit

THE ROMANOWSKY STAINS

The Romanowsky stains are mixtures of thiazine eosinates produced by the interaction of polychromed methylene blue (basic) with eosin (acidic) The neutral dye precipitated is redissolved in methyl alcohol and applied to the blood film After a length of time depending on the method the dyestuff is diluted with the appropriate buffer in water This results in an unstable solution in which the staining process is enhanced because of a temporary state of supersaturation Eventually however precipitation of insoluble compounds occurs The methyl alcohol in which the Romanowsky stains are dissolved fixes the blood films

Methylene blue on oxidation produces coloured compounds known as azures which combined with eosin produce red blue and purple compounds The Romanowsky stains vary in the amount of these coloured substances present Oxidation of methylene blue is termed polychroming and may be induced in many different ways Leishman s stain is produced by heating methylene blue at 65° C for 12 hours and then standing for 10 days to polychrome before eosinating Wright s stain differs only in that the methylene blue is polychromed for one hour in flowing steam Hasting s stain is polychromed methylene blue produced as for Wright s stain but then neutralized with acetic acid and unpolychromed methylene blue added before eosinating Jenner and May Grunwald stains are both produced by eosinating unpolychromed methylene blue and for practical purposes may be regarded as identical They are not true Romanowsky stains since the purple component is missing but polychroming does take place slowly on keeping Jenner and May Grunwald stains are not good by themselves since they are not good nuclear stains but cytoplasmic structures are demonstrated well and combined with Giemsa stain both stains give excellent results Giemsa stain is a mixture of azur eosinates methylene blue and glycerine with methyl alcohol The glycerine keeps the stain stable and enhances staining McNeal s tetrachrome stain is a similar mixture but without glycerine It does not keep well in alcoholic solution

The Romanowsky stains share the property that within limits the greater the stain dilution and the longer the staining period the better the results

DIFFERENTIAL LEUCOCYTE COUNTS

In a well spread film the distribution of leucocytes is such that a differential count made in a longitudinal strip is fairly representa

CHAPTER 4

STAINS AND STAINING

GENERAL

BLOOD FILMS

A WELL SPREAD and stained blood or marrow film is essential for accurate diagnosis Cover glass preparations are not recommended in spite of the better distribution of cells obtained because they are fragile and difficult to label and store Slides must be clean grease free and polished with lint free cloth The spreader consisting of another slide should have a smooth edge and one corner nicked off

THEORIES OF STAINING

Physical theories of staining assume that the dyestuff either penetrates the porous cell constituents is adsorbed on to them or is precipitated after penetration The chemical theory assumes that some cell and tissue constituents are acidic in nature and hence have an affinity for basic stains while others are basic in nature and hence have an affinity for acidic stains The theory also assumes that the tissue acids and bases are amphoteric and act as electrolytes dissolved in any fluid in which they are immersed although they are themselves insoluble Such a theory implies that the cell constituents act as acids or bases according to whether their isoelectric points are above or below the H ion concentration of the solution Stains for blood cells are used at *p*H 6 8 since in solutions of this reaction the nuclei take basic stains and the cytoplasm takes acidic stains Some workers prefer a buffer of *p*H 6 4 for blood and marrow films Malaria parasites show their characteristic pigment granules better when stained at *p*H 7 2 and histological sections require an acid buffer (*p*H 5 0) for optimal staining with Leishman s stain

The terminology used in haematology is based on the chemical theory Cell constituents staining with the basic part of the stain are said to be basophilic and those staining with the acidic part of the stain are said to be acidophilic or more commonly eosinophilic since eosin is the acidic stain used Those cell constituents staining between the two extremes are said to be neutrophilic

describe the degree of maturity of the neutrophils in a blood film and are merely descriptive of an imaginary release line dividing cells normally seen only in marrow from those seen in peripheral blood Normally this line would be drawn at the staff cell two lobed point but in infections the marrow pours younger forms into the blood stream The release line is then moved to the left—a left shift In megaloblastic anaemias older neutrophils predominate in the blood stream so the release line is moved to the right—a right shift

The Arneth Index

Arneth divided the granulocytes into five main classes and a number of subdivisions The myelocyte (M) a slightly indented nucleus (W) deeply indented nucleus (T) a rounded lobe (K) and a bent lobe (S) Depending on the number and shape of the lobes a cell could be classified for example as 2 K I S such a system is far too complicated for routine use The method of Cooke and Ponder ignores the shape of the lobes and merely uses five classes stressing however that the connecting link between lobes must be no more than a fine filament (Fig 14) The five classes are

Class I	No lobes i e myelocytes metamyelocytes and staff cells
Class II	Two lobes
Class III	Three lobes
Class IV	Four lobes
Class V	Five or more lobes

Normally non filamentous cells constitute 8 per cent of the total

Various indices may be calculated from the figures obtained by classifying 100 neutrophils The Arneth Index is the total of Classes I and II and half of Class III and is normally 59 Over this figure is a left shift and under 59 a right shift The weighted mean is obtained by multiplying together the number of each class and the number of cells in the class adding the whole and dividing by 100 Normally this is 2 74 and under this figure is a left shift and over a right shift

An uncommon inherited anomaly of neutrophil maturation known as the Pelger Huet anomaly gives the appearance of a left shift in the absence of sepsis In the fully inherited form the neutrophil nuclei are round or slightly indented while in the carrier state most of the cells are two lobed giving a *pince nez* appearance which is characteristic The condition has no association with disease

tive of the whole The edges of the film however do show a higher percentage of neutrophils and monocytes Using the strip method 200 cells should be counted additional strips being counted if necessary to reach this figure Counts which show a large number of nucleated red cells should have these included in the differential count in order that adjustment may be made in the total leucocyte count which is in effect a nucleated count A separate estimation is then made for the differential white cell count Some centres where many patients are being irradiated use the battlement method of differential counting This implies counting three edge fields then two transverse fields followed by three longitudinal fields and back to the edge again This method weights the count too heavily in respect of neutrophils but since irradiation may produce a leucopenic state due to reduction in the absolute numbers of lymphocytes this technique is regarded as giving some warning of this complication of the treatment

NUCLEAR INDICES

Classification of neutrophils (Fig 14) on the degree of nuclear lobulation is based on the assumption that such cells become more lobulated as they age Nuclear indices therefore give some indication of bone marrow activity The terms right and left shift are used to

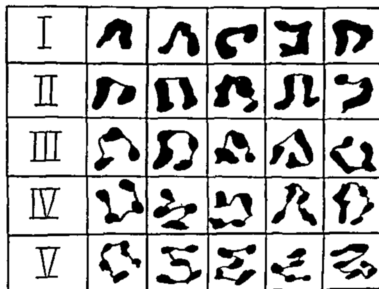


FIG 14 —The classification of neutrophils or number of lobes

haemoglobin may be roughly gauged from the staining appearances Abnormally thin red cells are seen in iron deficiency and other anaemias and are termed leptocytes Such cells may have only a thin stained area at the periphery and are sometimes called pessary cells This central pallor or hypochromia in a normal sized red cell is indicative of iron deficiency Bowl shaped or Mexican hat cells seen in some haemolytic anaemias show a central stained spot and an outer ring of stained material They are termed target cells The spherocyte seen in some haemolytic anaemias is a red cell which has gained in thickness at the expense of its diameter, and may be recognized in a stained film as a small densely stained cell

Granulocyte staining reaction

The granulocytes normally stain specifically in respect of their granulations but in septic and toxic conditions large black granules make their appearance in the neutrophil cytoplasm This is termed toxic granulation and is usually accompanied by vacuolation of the cytoplasm The cytoplasm itself may undergo degeneration with the appearance of irregular blue areas termed Döhle or Amato bodies They are seen in many conditions but do not appear in German measles In toxic conditions the cells may become so badly damaged that they are ruptured during the preparation of the film The neutrophils are then said to show toxic degenerative changes

The cells of the granulocyte series with the exception of the basophil contain within their cytoplasm an enzyme termed a peroxidase In the presence of this enzyme benzidine is oxidized to a black compound by hydrogen peroxide The test is used to differentiate cells of the granulocyte and other series but its value is small The early myeloblast is peroxidase negative (Oxydasen schwund of Jurich) but later cells of the granulocyte series contain increasing amounts The lymphocyte series is uniformly negative but monocytes sometimes contain a few positive granules Some authorities believe this to be due to phagocytosis of granules derived from destroyed granulocytes The large black granules found in toxic neutrophils are peroxidase negative Since benzidine is no longer being manufactured substitutes have been suggested for this test The *alpha* naphthol and *ortho* toluidine methods are just as satisfactory but there seems no doubt that the reaction of the cells to fat stains provide the same information The peroxidase positive granules are sudanophilic that is, they stain with Sudan III and allied stains The Sudan black B method (*see* page 58) is the method of choice although batches of dyestuff vary Mitochondria and Auer bodies are also stained by Sudan black B

The Schilling divisions

Schilling divided the granulocytes into myelocytes juveniles (metamyelocytes) stabzellen (non lobed degenerative forms) and segmented neutrophils but he also included the other leucocytes in the count

Schilling distinguished between a regenerative shift in which there is a high total white cell count with an increase in younger cells and a degenerative shift in which there is a low white cell count with an increase in stab cells without myelocytes or juveniles and increase in monocytes with disappearance of eosinophils and basophils The stab cell of Schilling is a neutrophil with small deeply staining twisted nucleus a cell which does not develop further than this stage

SEX OF NEUTROPHILS

It is possible to determine the true sex of an hermaphrodite by examination of a blood film That part of the nuclear chromatin which is concerned with sex determination forms a club of distinctive shape on the last or next to last lobe of the neutrophils (frontis piece) The material is either round or racquet shaped and if present in more than 6 in 300 cells examined is regarded as indicative of female sex

STAINING REACTIONS

Red cell staining reaction

The normal red cell fully saturated with haemoglobin stains uniformly pink with Romanowsky stains but the more immature cells stain with varying degrees of basophilia Such cells contain within their cytoplasm mixtures of haemoglobin and ribonucleic acid the earliest cells having only ribonucleic acid and protein A cell showing both pink and blue areas is said to show poly chromasia or polychromatophilia The basophilic material may appear as dots sometimes fine and numerous at other times coarse and scanty and this is termed punctate basophilia or basophilic stippling The presence of ribonucleic acid in the cytoplasm is a sign of immaturity whether it is diffuse or aggregated and cells containing the material are in fact reticulocytes the reticulum being demonstrated by supravital staining (*see* page 60) Basophilic stippling occurs in many anaemias and in poisoning with heavy metals The degree of stippling in lead workers is taken as some indication of exposure to the toxic agent

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CHROMATIN AND NUCLEIC ACIDS

The term chromatin has become synonymous with nuclear material staining with basic dyes but chromatin is not a single entity The nucleolus contains ribonucleic acid (RNA) and pro teins which in association with heterochromatin forming the edge of the nucleolus control the maturation of the cell Basophilic cytoplasm also contains RNA and these three sources of the material are concerned with the production of the enzyme processes associated with the cytoplasmic granules and the synthesis of haemoglobin RNA normally decreases as the cell matures but in leukaemia and pernicious anaemia the level of RNA remains high particularly in the heterochromatin Basophilic cytoplasm or ergastoplasm of erythroblasts disappears as haemoglobin is formed Mitochondria contain RNA and the unstained patches termed paraplasm or hyaloplasm seen in the cytoplasm of immature white cells are believed to be the reflecting image of underlying mitochondria

Most of the nuclear chromatin consists of desoxyribonucleic acid (DNA) Chromosomes consist of this nucleic acid as do true nuclear remnants such as Howell Jolly bodies Non basophilic cytoplasm also contains DNA

Specific staining

Histological staining procedures have been adapted to demon strate nucleic acids in blood cells Unna Pappenheim staining of plasma cells if certain conditions are complied with is specific for RNA and DNA Ribonucleic acid persists in the cytoplasm of mature plasma cells and is stained with pyronin Y though not all batches of stain are suitable DNA is stained with methyl green in the same technique The pyronin is specific for RNA if the tech nique is controlled by ribonuclease extraction The enzyme removes the RNA so that the control slide is unstained Treatment with a 1 1 000 aqueous solution of crystalline ribonuclease for 1 hour at 37 C is sufficient to remove the RNA Bile salt will also remove RNA as will 10 per cent perchloric acid at 4 C but perchloric acid 5 per cent at 60 C will remove both RNA and DNA *Trichloracetic acid 4 per cent at 90 C removes both RNA and* DNA while DNA can be specifically removed by desoxyribonuclease These extraction techniques are merely confirmatory evidence that the staining procedure used is actually demonstrating RNA and DNA

Use of Schiff s reagent—The Feulgen reaction is based on the release of aldehydes from the desoxypentose sugar of DNA by mild acid hydrolysis The aldehydes are detected by Schiff s reagent

(leuco fuchsin) which gives a reddish purple colour due to the formation of a quinonoid compound in the presence of aldehydes The method is extremely useful in that nucleoli are shown up so well—even where using a Romanowsky method they cannot be seen at all Red cells do not stain nor does cell cytoplasm unless a counterstain is used Nuclei of leucocytes megakaryocytes and erythroblasts stain pinkish and nucleoli are clearly defined unstained areas The primitive cells staining a more intense pink are termed Feulgen positive The acid hydrolysis is important in this technique the time depending a great deal on the fixative used

The Schiff reagent is prepared by bubbling SO_2 through a solution of basic fuchsin and hydrochloric acid but it is far less trouble to buy the reagent ready made (George T Gurr Ltd)

Supravital staining of leucocytes is a technique aimed at staining the living cell and has largely been superseded by phase contrast microscopy Janus green B and vital neutral red are used in supravital techniques to stain mitochondria and neutral red vacuoles The stained cells are observed for motility on a warm stage

RED CELL INCLUSIONS

Red cells in disease may show a great variety of inclusions Howell Jolly bodies are true nuclear remnants formed by karyor rhexis of the erythroblast nucleus One or more may be present in a single cell They stain a purple colour with Romanowsky stains and are Feulgen positive They are found in many anaemias and after splenectomy or where the spleen is failing to function The Cabot ring stains a reddish purple and appears as a ring or figure of eight It is not a true structure and is probably an artefact produced as a result of shrinkage of an abnormal cell The Cabot ring is found in many anaemias but is not diagnostic of any con dition Pappenheimer bodies usually occurring as a single dot of iron containing material are common after splenectomy or in splenic atrophy They stain purple with Romanowsky stains Some parasites also present themselves in red cells malaria being an example

OTHER COMMENTS

Much information can be gained by inspection of the stained film first by low power and then by the high power lens of the microscope Anisocytosis is a term used to describe variation in red cell size and poikilocytosis a term used to describe the presence of pear shaped cells Macrocytosis in the absence of anisocytosis may be an important finding in early megaloblastic anaemias Burr cells are crenated red cells with a stalk and their presence is of

diagnostic significance in renal disease Red cells may be oval in rare cases This is an inherited condition sometimes associated with a haemolytic anaemia Crescent cells are reddish purple, half moon shaped bodies which are red cell stromas They are spreading artefacts resulting from an abnormal cell Senile cells may be recognized in blood films and if present in large numbers may be indicative of a leucocyte dyscrasia Basket cells are senile neutrophils and smudge cells are senile lymphocytes The presence absence or abnormality of platelets must also be noted

TECHNIQUE

PREPARATION OF BLOOD FILMS

Methods of preparing blood films are as follow

Thin films

(1) Place a small drop of blood at one end of a slide
(2) Move the spreader back at an angle of 45 degrees until it meets the blood
(3) When the blood has run along the edge of the spreader move the spreader forward in a smooth rapid movement pulling the blood after it

The film should be of such a thickness that it dries immediately both margins should be clear of the slide edges and it should have no tails

Thick films first method

(1) Coat a draughtsman s ruling pen with a silicone preparation so that the blades are non water wettable
(2) Dip the pen in the blood and draw lines 0 25 mm in width on a slide Thicken the lines several times
(3) Allow to dry at room temperature

This method may be used either for the demonstration of scanty malaria parasites or for performing differential white-cell counts It is not a good method for conditions showing abnormal cells but in disorders where the neutrophil lymphocyte ratio is of prime importance (irradiation treatment with thiouracil and so on) it saves a great deal of time The cell distribution lends itself to accuracy in the count and the red cells are lysed during staining

Thick films second method

(1) Place a large drop of blood in the centre of a slide
(2) With the corner of another slide spread the blood until the hands of a watch can be seen through the smear
(3) Cover with a Petrie dish lid and allow to dry

Note Unstained blood films are covered to prevent access of flies

PREPARATION OF LEISHMAN S STAIN

Three methods of preparing Leishman s stain from dry powder are given below but 0 15 g of powder or crystals to 100 ml of methyl alcohol is used in each case However, batches of Romanowsky stains vary, so that it may be necessary to adjust the amount of solid stain by an amount determined on a pilot sample

(1) Place the weighed crystals in a stoppered flask and add pure methyl alcohol Shake the flask at intervals throughout the day The stain is ready for use the next day
(2) Triturate the crystals in a mortar with pure methyl alcohol pouring off the supernatant and adding more alcohol until the crystals are all dissolved Stand for 24 hours before use
(3) Reduce the crystals to a fine powder in a mortar and add pure methyl alcohol stirring until all the powder is dissolved Stand for 24 hours before use

Jenner and May Grunwald stains may be prepared in exactly the same way as Leishman but 0 3 g of dry stain is used per 100 ml of methyl alcohol

PREPARATION OF GIEMSA STAIN

Giemsa stain is prepared as follows

(1) Grind 0 75 g of dry stain in a mortar with 25 ml of pure glycerine until a smooth paste is obtained
(2) Stir 75 ml of pure methyl alcohol thoroughly into the paste and transfer to a stoppered bottle
(3) Place the reagent in the incubator at 37 C for 24 hours shaking at frequent intervals

STAINING WITH LEISHMAN S STAIN

Methods of staining with Leishman s stain are as follow

Thin films of blood or marrow

(1) Cover the dry unfixed film with undiluted stain and allow to remain 30–60 seconds The shorter time is used in hot weather
(2) Add to the undiluted stain on the slide twice the volume of buffered distilled water *p*H 6 8 and mix by gentle rocking Allow to stain for 10 minutes
(3) Flood off the stain with distilled water and wash until the film is pink
(4) Drain and dry at room temperature

Thick films for malaria parasites

(1) Pour on Leishman s stain diluted with five parts of buffered dis tilled water *p*H 7 2 and allow to stain for 30 minutes
(2) Wash off gently with distilled water
(3) Drain and dry at room temperature

Sections

Tissues for the demonstration of blood cells are usually fixed in a mercury containing fixative The mercuric deposit must be removed by treatment with alcoholic iodine followed by 5 per cent sodium thiosulphate before staining

(1) Deparaffinize with xylene then take through alcohol to water
(2) Remove mercuric deposit
(3) Wash the sections in running water followed by distilled water
(4) Flood the slide with a mixture of Leishman s stain one part and two parts of buffered distilled water *p*H 5 0 and warm until steam rises Allow to stain for 10 minutes
(5) Wash rapidly with 50 per cent methyl alcohol in *p*H 5 0 buffer
(6) Transfer to absolute methyl alcohol
(7) Clear in xylol and mount in neutral mounting medium

Wright s stain may be substituted in all the foregoing methods

STAINING WITH GIEMSA STAIN

Methods of staining with Giemsa stain are as follow

Thin films of blood or marrow

(1) Fix the film in methyl alcohol for 5 minutes
(2) Dilute the stain 1 10 with buffered distilled water *p*H 6 8 and stand the films in a Coplin jar for 20 minutes (this time may need to be prolonged to demonstrate adequately parasites such as spirilla and trypanosomes)
(3) Rinse in distilled water
(4) Drain and dry at room temperature

Thick films for malaria parasites

(1) Stain the dry unfixed film in a staining jar for one hour with Giemsa diluted 1 50 with buffered distilled water *p*H 7 2
(2) Differentiate in distilled water for a few minutes
(3) Drain and dry at room temperature

Sections

(1) Deparaffinize with xylene then take through alcohol to water
(2) Remove the mercuric deposit
(3) Wash the sections in running water followed by distilled water
(4) Stain overnight in a staining jar of Giemsa diluted 1 25 with distilled water buffered to *p*H 6 8
(5) Rinse in distilled water
(6) Differentiate in 1 1 500 acetic acid controlling microscopically until the nuclei are blue and the eosinophil granules red
(7) Rapidly rinse in distilled water

(8) Dehydrate in absolute alcohol
(9) Clear in xylol and mount in neutral mounting medium

Note A scum forms in diluted Giemsa solutions on standing This should be flushed off before removing the slides from the jar

PANOPTIC STAINING METHOD (JENNER GIEMSA OR MAY GRUNWALD-GIEMSA COMBINED STAINING METHOD

The panoptic staining method for blood and marrow films is detailed hereunder

(1) Stain the dry unfixed films for 3 minutes in undiluted Jenner or May Grunwald stain
(2) Add an equal volume of buffered distilled water *p*H 6 8 and stain for 2 minutes
(3) Rinse with buffer and allow to remain one minute
(4) *Stain with 1 10 Giemsa diluted with buffered distilled water p*H 6 8 for 10-15 minutes
(5) Rinse in distilled water
(6) Drain and dry at room temperature

PREPARATION OF FIELD S STAIN

Stain A	Methylene blue	1 3 g
	Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	12 6 g
	Potassium dihydrogen phosphate (KH_2PO_4)	6 25 g
	Distilled water	500 ml

Dissolve the methylene blue and the disodium hydrogen phosphate in 50 ml of distilled water and boil the solution almost to dryness in a water bath to polychrome the methylene blue Then add the potassium dihydrogen phosphate and 500 ml of freshly boiled distilled water Stir to dissolve the stain set aside for 24 hours and filter Filter again before use

Stain B	Eosin W S	1 3 g
	Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	12 6 g
	Potassium dihydrogen phosphate (KH_2PO_4)	6 25 g
	Distilled water	500 ml

Dissolve *the phosphates in warm freshly boiled distilled water and* add the dye Allow the solution to stand for 24 hours and then filter

STAINING WITH FIELD S STAIN

Methods of staining with Field s stain are as follow

Thick films for malaria parasites

(1) Dip *the dry unfixed film in Stain A for a few seconds*

(2) Rinse in buffered distilled water pH 7 0 for a few seconds
(3) Dip the film in Stain B for a few seconds
(4) Rinse rapidly in buffered distilled water pH 7 0
(5) Drain and dry at room temperature

Reticulocyte counts

(1) *To oxalated blood add a crystal or two of brilliant cresyl blue* sufficient to colour the blood Mix well for one minute
(2) Make films from the stained blood
(3) Examine under the high power of the microscope using a squared eyepiece and count 2 000 cells This is not a laborious procedure and in a well spread film the statistical error of reticulocyte counts of more than 5 per cent is not large

To save time the reticulocytes alone may be counted until 100 have been recorded The total number of red cells in several fields is then counted and the probable number present in the area containing 100 reticulocytes calculated The reticulocyte percentage is then calculated from this figure

Peroxidase method

(1) Fix fresh blood or marrow films in 90 per cent ethyl alcohol for 5 minutes
(2) Wash in running water followed by distilled water
(3) Stain upside down on a staining plate in a mixture of 10 ml phos phate buffer pH 7 2 4–5 drops of 0 75 per cent benzidine in acetone 2 drops of 20 vols hydrogen peroxide Depending on the cellularity of the film the staining time varies from 5 to 15 minutes
(4) Wash thoroughly in running water followed by distilled water
(5) Superstain with Giemsa 1 6 diluted with buffer pH 6 8 for 15 minutes
(6) Rinse and dry at room temperature

The benzidine solution may be used immediately but it will be noted that it darkens on standing The final benzidine reagent in buffer should be a straw colour The granules stain yellow to brown

STAINING WITH SUDAN BLACK

Details of the Sudan black reagents and staining methods are as follow

Reagents

Formol alcohol	40 per cent formaldehyde	10 ml
	95 per cent ethanol	90 ml
Sudan black B	Saturated solution of Sudan black B in 70 per cent alcohol This does not keep well	

Methods

(1) Fix film of blood or marrow in formol alcohol for 5 seconds
(2) Wash in distilled water and dry
(3) Place the film face downwards on thin glass supports in a Petrie dish and pour in solution of Sudan black B until level reaches the slide
(4) Place Petrie dish in incubator at 37° C for 30 minutes
(5) Remove the film and without washing counterstain with May Grunwald for 3 minutes
(6) Drain without washing
(7) Cover with 1 20 Giemsa and leave for 15–40 minutes
(8) Wash in distilled water and dry

UNNA PAPPENHEIM STAIN FOR RIBONUCLEIC ACID

The method of using the Unna Pappenheim stain for ribonucleic acid is as follows

(1) Make films of blood or marrow and without drying place in Heidenhain s Susa to fix for one hour
(2) Rinse in water and treat with Gram s iodine solution for 5 minutes
(3) Rinse in water and treat with 1 per cent sodium thiosulphate for 5 minutes
(4) Wash thoroughly in running water followed by distilled water
(5) Stain the films for one hour in pyronin methyl green stain

Pyronin Y (Gurr)	0 3 g
Methyl green	0 7 g
Glycerine	20 ml
Absolute ethanol	2 5 ml
0 5 per cent phenol in distilled water to 100 ml	

Grind the stains in a mortar with the glycerine and ethanol and then add the phenol solution Boil the solution for 2 minutes and filter

(6) Rinse in distilled water
(7) *Dehydrate in tertiary butanol*
(8) Clear in xylol and mount in neutral mounting medium

FEULGEN STAIN FOR DESOXYRIBONUCLEIC ACID

The method of using the Feulgen stain for desoxyribonucleic acid is as follows

(1) Fix blood or marrow films in Susa for one hour
(2) Remove mercuric deposit as in Unna Pappenheim method
(3) *Hydrolyse in hot (56 -60 C)* hydrochloric acid[1] for 4 minutes (This time may need adjustment)
(4) Dip the slides in cold acid of the same strength

[1] 82 5 ml conc HCl S G 1 19 made up to 1 litre with distilled water

(5) Wash in distilled water
(6) Stain for 2 hours in Feulgen fuchsin sulphurous acid Dissolve 1 g of basic fuchsin in 200 ml of boiling distilled water and filter when the solution has cooled to 50° C Allow the solution to cool to 25 C and add 20 ml of N HCl and 1 g anhydrous sodium bisulphite Sulphur dioxide is evolved and the solution turns yellow The reagent must not be used for 24 hours and should be stored in a stoppered bottle in the dark
(7) Wash for 15 minutes in three successive jars of a mixture of 10 ml of 10 per cent aqueous anhydrous sodium bisulphite 10 ml of N HCl and distilled water to 200 ml
(8) Rinse in running water for 10 minutes
(9) Rinse in distilled water
(10) Counterstain for a few seconds in 1 per cent aqueous light green
(11) Rinse in distilled water drain and dry

SUPRAVITAL STAINING

The reagents and method used in supravital staining are as follow

Reagents

Stock neutral red solution 0 25 per cent neutral red chloride in neutral absolute ethanol

Stock Janus green B 0 4 per cent in neutral absolute ethanol The proportions of stains used may be varied and the thickness of the stain on the prepared slide may also be adjusted for the number of leucocytes

Methods

(1) Immediately before use mix 1 75 ml of stock neutral red solution with 0 07 ml of stock Janus green solution and 10 ml neutral absolute ethanol
(2) Flood perfectly clean slides with the mixed stain pour off the excess and drain dry at 37 C The slides may be packed together and stored
(3) Place a drop of blood on a coverslip and invert on one of the pre pared slides
(4) Seal the edges of the preparation with a mixture of paraffin wax and Vaseline and incubate for 15 minutes at 37° C

CHAPTER 5

HAEMATOLOGICAL VALUES

GENERAL

VALUES AND INDICES

HAEMATOLOGICAL values and indices are only as accurate as the data from which they are calculated The error of the red-cell count can be greatly minimized using electronic methods and counting large numbers of cells but the inaccuracies inherent in the usual counting chamber techniques impose limitations on any classification of anaemias on the basis of values and indices

COLOUR INDEX

The colour index which expresses the mean haemoglobin content of a single red cell compared with the content of a normal red cell, was originally used to classify the anaemias For the purposes of calculation the normal red cell count is assumed to be 5 0 millions per c mm irrespective of age or sex and the normal haemoglobin to be 14 5 g per 100 ml blood equivalent to 100 per cent This haemoglobin value avoids errors arising from the use of other standards which must be corrected to this figure Values obtained from modern Haldane and Sahli instruments may be used without correction since the standards approximate this arbitrary normal The colour index is obtained by dividing the haemoglobin percentage by the red cell percentage (the first two figures of the red cell count multiplied by two)

The information yielded by the colour index is small and even misleading An index of less than 1 0 may be produced in iron deficient states where the red cell is normal in size or larger or smaller than normal A high colour index however is always associated with macrocytosis

ABSOLUTE VALUES

The experienced worker can obtain from an estimation of haemoglobin and a careful examination of a stained blood film much of the information necessary to classify an anaemia but for record purposes some reproducible scheme is necessary The

system of absolute values is far from absolute but it does provide figures which may be recorded and within the limits of error of red cell counts may be reproduced in different laboratories

The absolute values used in haematology are as follows

(*a*) Mean corpuscular haemoglobin (M C H)
(*b*) Mean corpuscular haemoglobin concentration (M C H C)
(*c*) Mean corpuscular volume (M C V)
(*d*) Mean corpuscular average thickness (M C A T)

These values are calculated from the haemoglobin estimation red cell count packed cell volume and mean diameter of the red cells and may be conveniently read from the haematological slide rule (*Fig 15*) *The basic calculations however should not be* forgotten

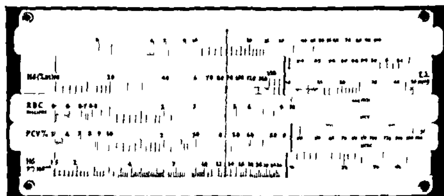


FIG 15 —Haematological slide rule (Camlab) (*Reproduced by courtesy of Camlab (Glass) Limited Cambridge England*)

Packed cell volume

The packed cell volume (P C V) is obtained by centrifuging oxalated or heparinized blood in the Wintrobe haematocrit under standard conditions The haematocrit is a stout glass tube 110 mm long with a 2 5–3 0 mm bore and graduated from 0–100 in centimetres and millimetres The volume of blood contained in the graduated part of the tube is approximately 0 8 ml The normal range of packed cell volume in men is 40–56 per cent in women 35–48 per cent in children 32–44 per cent and in cord blood of infants 49–60 per cent Such results are reproducible to within 1 0 per cent but the blood must be well mixed and aerated and centrifuged for 60 minutes at 3 000 r p m in a centrifuge with a radius of 15 cm Even under these conditions some plasma is trapped in the

packed cell mass but is important only in blood and plasma volume estimations Aerated blood gives slightly lower figures than venous blood

The cells are packed in three layers the lowest being red cells At the top of this layer may be seen a thin dark red band of red cells whose oxyhaemoglobin has been reduced by the activity of the leucocyte layer which is superimposed The leucocyte layer termed the buffy coat is reddish grey and is surmounted by a thin creamy white layer of platelets The colour of the supernatant plasma varies from a pale watery colour in severe anaemia to a deep icteric tinge in jaundice Carotin and lutein may colour the plasma deeply Carotinaemia may occur in myxoedema, a disorder sometimes accompanied by a macrocytic anaemia and the same pigment may appear after a meal of eggs or carrots

The packed cell volume may be determined on capillary blood very rapidly, using only small amounts of material in the micro haematocrit centrifuge (Fig 16) which develops 12 000 G [1]

Mean corpuscular haemoglobin (M C H)

The mean corpuscular haemoglobin value expresses the average

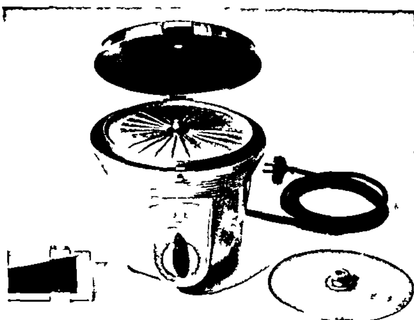


Fig 16 —Microhaematocrit centrifuge the grooves in which the capillary tubes lie are seen in the centrifuge head (*Reproduced by courtesy of Hawksley and Son Ltd London*)

[1] $G = 0\ 00001118 \times N \times r^2$ where N = r p m and r = radius

haemoglobin content of a single red cell in micro micrograms the normal range being 27–32$\mu\mu$g

$$\text{M C H} = \frac{\text{Haemoglobin in grammes per 1 000 ml blood}}{\text{R B C in millions per c mm}}$$

The reason for including in the calculation the amount of haemoglobin per litre of blood may not be immediately apparent If the calculation is made from first principles however, it will be found that this is merely a convenient artifice, for example haemoglobin 14 5 g per cent, R B C 5 mill /c mm
Haemoglobin of cells in 100 ml blood is 14 5 $\times$ 10^{1} $\mu\mu$g
Number of red cells in 100 ml blood is 5 $\times$ 10^{11}

$$\text{M C H} = \frac{14\ 5 \times 10^{12}}{5 \times 10^{11}} = \frac{14\ 5 \times 10}{5} = \text{weight of haemoglobin per single red cell in micro-micrograms}$$

The M C H is not a useful absolute value and in fact gives no more information than the colour index which may be calculated by dividing the M C H by the normal M C H

Mean corpuscular haemoglobin concentration (M C H C)

The mean corpuscular haemoglobin concentration value is calculated from two accurate and reproducible observations and is a reliable index of iron deficiency It measures the number of grammes of haemoglobin in 100 ml of red blood cells and the normal of 32–38 per cent is said to be fully saturated The term desaturated is applied to an anaemia characterized by a low M C H C

$$\text{M C H C} = \frac{\text{Haemoglobin in grammes per 100 ml of blood}}{\text{Packed cell volume per 100 ml of blood}} \times 100$$

The calculation actually relates the weight of haemoglobin to the volume of red cells contained in 100 ml of blood and expresses the result as a percentage Since the red cell count is not involved this must be regarded as the most accurate of the absolute values

Mean corpuscular volume (M C V)

The mean corpuscular volume value measures red cell size by volume and anaemias may be classified as normocytic microcytic or macrocytic on the result Since the red cell count is involved great care must be taken to minimize the errors of this technique

$$\text{M C V} = \frac{\text{Packed cell volume of 1 000 ml of blood}}{\text{R B C in millions per c mm}}$$

The normal range is 78–94 cubic microns

Once again the reason for the use of the measure 1,000 ml of blood becomes obvious when calculations are made from first principles, for example, P C V = 45 per cent R B C = 5 0 millions per c mm

P C.V in 100 ml blood = 45×10^{12} cubic microns
R B C in 100 ml blood = 5×10^{11}

$$M C V = \frac{45 \times 10^{12}}{5 \times 10^{11}} = \frac{45 \times 10}{5} = \text{volume of a single cell in cubic microns}$$

Mean corpuscular average thickness (M C A T)

The mean corpuscular average thickness value by itself is not much used It is determined by assuming the red cell to have the shape of a squat cylinder by balancing concavities against con vexities

$$M C A T = \frac{M C V}{\left(\frac{M C D}{2}\right)^2} \quad M C D = \text{Diameter in microns}$$

The normal range is 1 7–2 5μ

DIAMETER THICKNESS RATIO

The diameter thickness ratio is not an absolute value but is regarded as a good index of spherocytosis The M C A T (*see* above) may alter in disease without alteration in cell shape but alterations in the diameter thickness ratio occur only in definite conditions The normal range of the diameter thickness ratio is 2 4–4 2 1 the average being 3 4 1 Values below 2 4 1 indicate spherocytosis and values above 4 2 1 a flat cell

MEAN CORPUSCULAR DIAMETER

The mean corpuscular diameter value may be determined in many different ways including direct measurement by means of a micrometer eyepiece photography at a known magnification and subsequent measurement of a random selection of cells halometry or even by mixing normal blood with the patient s cells and com paring microscopically The last technique has been modified by using chicken blood as a control

Halometry

Halometry measures the modal mean that is the size common to the largest number of cells and is a routine haematological

procedure *The principle of the method is that* a blood *film* acts as a diffraction grating in that when a parallel beam of white light is passed through the film the light is diffracted around each cell the degree of diffraction varying with the cell diameter To the human eye this appears as a single halo around the light source surrounded by the spectral colours from violet to red and fainter colours outside the red Too much anisocytosis produces an indistinct spectral pattern since the final halo is the result of blending of all the diffrac tion rings *The size of the halo varies directly with the distance of* the film from the light source and inversely with the size of the red cell Since staining may increase the cell size by deposition of stain, *unstained preparations are used for halometry*

Halometric measuring methods —Many different instruments have been elaborated for halometric measurements including those com paring normal and abnormal halos and modifications using mono chromatic light but the apparatus most used in this country is that of Eve in which the light source is split in two by means of a mirror so that a double halo is produced The distance between film and light source is adjusted until the red rings just touch and from the angle formed by the two lights and the film the red cell diameter is calculated The instrument is calibrated in that angles are corre lated with red cell diameters

RED CELL DIAMETER DISTRIBUTION CURVE (PRICE JONES)

Accurate measurement of a series of red cells taken at random provides the data for a statistical analysis Between 200–500 cells are usually measured and a curve drawn plotting cell size against numbers of cells in each group Price Jones pointed out that the normal distribution curve can be split into two curves representing the upper and lower limits of normal red cell size (Fig 17) From such curves the percentage of microcytosis or macrocytosis or both, may be obtained and anisocytosis is indicated by a spreading of the base *and flattening of the peak Price Jones statistical procedure enables* the arithmetic mean (M) standard deviation (σ) and the coefficient of variation (v) of the red cell diameters to be calculated The arithmetic mean is the mean diameter of the number of cells measured and the standard deviation is a measure of anisocytosis The coefficient of variation is the standard deviation expressed as a percentage of the arithmetic mean It should be noted that the modal mean corresponds with the arithmetic mean only in the ideal curve The calculations are shown in the second part of this chapter

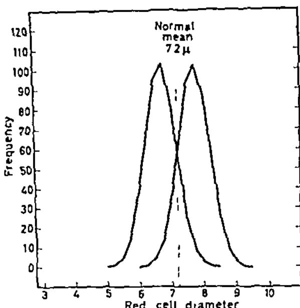


FIG 17 —Red-cell size distribution curves (Price Jones) showing upper and lower limits of normality

TECHNIQUE

DETERMINATION OF PACKED CELL VOLUME (P C V)

Methods to be used in the determining of packed cell volume (P C V) are as follow

Venous blood

(1) Collect venous blood into Heller and Paul mixture
(2) Mix and aerate thoroughly
(3) With a capillary pipette fill haematocrit to the 100 mark
(4) Centrifuge at 3 000 r p m for 60 minutes in centrifuge with radius of 15 cm

Capillary blood using the micro haematocrit centrifuge

(1) Collect blood from ear or finger into heparinized capillary tube to within 1 inch of the end
(2) Seal the unfilled end in Bunsen flame
(3) Place the tube in a numbered slot in the centrifuge head with sealed end outward
(4) Replace and screw down metal cover of centrifuge
(5) Close the hinged lid and set automatic time switch to 5 minutes
(6) After centrifuge stops remove tubes and read by placing against white paper and marking positions of cell and plasma levels [1]

[1] A reader supplied by Hawksley and Son Ltd facilitates the reading of microhaematocrits

THE MEASUREMENT OF RED CELL DIAMETER (PRICE JONES)

Red cell diameter measurements are obtained by the following method

(1) Remove the mirror from a monocular microscope and place the instrument in the horizontal position
(2) Direct a beam of light from a Pointolite or similar lamp through the condenser
(3) Shield the microscope and use in a darkened room
(4) Place a micrometer slide on the stage and using the 2 mm oil immersion objective focus the ruling
(5) Fix the microscope mirror in a burette stand so that the plane sur face is in line with the ocular and the ruling projected on to a sheet of white paper on the bench
(6) Adjust the distance of the mirror from the ocular until 0 01 mm of the micrometer ruling is projected as 10 mm on the sheet (mag nification × 1 000) This gives a value of $1\mu = 1$ mm
(7) Replace the micrometer slide with a thinly spread blood film stained 2 minutes with Jenner rinsed dry and superstained 2 minutes with 0 25 per cent aqueous eosin
(8) Measure the diameters of 250 cells selected at random by measuring the longest and shortest axis to the nearest 0 5 mm and taking the mean of the two readings This results in a series of figures which are divided into classes varying by 0 25 mm which on the magnifi cation employed is equivalent to 0 25μ

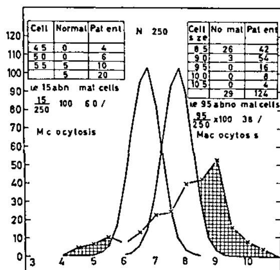


FIG 18—Red-cell size distribution curve showing spreading of base and flattening of peak The calcula tions of microcytosis and macrocytosis are shown in the boxes

CALCULATION OF MACROCYTOSIS AND MICROCYTOSIS

To obtain a calculation of macrocytosis and microcytosis the following method is used

(1) Using the data obtained by the foregoing technique construct a red-cell diameter distribution curve by plotting diameters on the abscissa and numbers on the ordinate

(2) Sum the numbers of cells outside the normal curve to the left of the normal curve subtract those falling within normal limits and express as a percentage of the total number of cells measured = microcytosis Repeat the procedure with those cells to the right of the normal curve = macrocytosis (Fig 18)

TABLE III

CALCULATION OF MACROCYTOSIS AND MICROCYTOSIS

I	*II*	*III*	*IV*	*V*
Red cell diameter	*Frequency (f) No of cells in each class*	*Deviation (ξ) in 0 25μ from arbitrary mean*	$f\xi$	$f\xi^2$
6 0	12	− 4	− 48 } − 194	192
6 25	20	− 3	− 60	180
6 5	26	− 2	− 52	104
6 75	34	− 1	− 34	34
7·0	42	Arbitrary mean		
7 25	38	+ 1	38 } + 316	38 $\frac{1\ 658}{N} = \frac{1\ 658}{250}$
7 5	26	+ 2	52	104
7 75	18	+ 3	54	162 = 6 632 i e square of deviation from arbitrary mean in terms of 0 25μ
8 0	14	+ 4	64	256
8 25	12	+ 5	60	300
8 5	8	+ 6	48	288
	N = 250			1 658

Column IV:

316 − 194 = 122

$\frac{122}{N} = \frac{122}{250} = 0\ 488$

0 488 (0 25) = 0 122

M = 7 0 + 0 122 = 7 122μ

Column V:

Difference between arbitrary mean and arithmetic mean in 0 25μ = 0 488

0 488² = 0 2381

σ² in 0 25μ = 6 632 − 0 2381 = 6 3939

σ in 0 25μ = $\sqrt{6\ 3939}$ = 2 53

σ = 0 63μ

$v = \frac{0\ 63}{7\ 122} \times 100$ = 8 8 %

To obtain the arithmetic mean the figures in columns I and II of Table III can be multiplied together, summed and divided by 250, but it is more convenient to pick an arbitrary mean somewhere near the probable arithmetic mean This can be obtained by inspection Column III then shows how each class differs from the arbitrary mean in terms of 0 25μ If these two columns, II and III, are then multiplied together, summed and divided by 250, a figure will be obtained which after conversion to microns, is either added to or subtracted from the arbitrary mean depending on the sign in front of it, to obtain the arithmetic mean The figure is converted to microns by dividing by four The last column is a list of figures derived from the frequency and the square of the deviations Squaring deviations is a statistical method of making them more obvious If these deviations are squared multiplied by the fre quencies summed and then divided by 250, the resultant quotient represents the square of the deviation from the arbitrary mean in 0 25μ Standard deviation however, is the deviation from the arithmetic mean so it is necessary to adjust this figure by subtracting from it the square of the difference between arithmetic and arbitrary mean (found in column IV) The square root of this figure is the standard deviation in terms of 0 25μ which is converted to microns simply by dividing by four

The normal arithmetic mean is (M)	6 7 –7 7μ
The normal standard deviation is (σ)	0 35–0 56μ
The normal coefficient of variation is (v)	5 3–7 3 per cent

CHAPTER 6

THE INVESTIGATION OF ANAEMIC AND POLYCYTHAEMIC STATES

ANAEMIC STATES

DEFINITION

ANAEMIA is defined as a loss of the normal balance between productive and destructive blood processes due to diminution of blood volume or deficiency in the number of red cells haemoglobin or both The condition is usually a manifestation of an underlying disease process rather than a primary disorder of the erythron which is comparatively rare Anaemias may be broadly divided into four groups

(*a*) Anaemias of unknown cause
(*b*) Dyshaemopoietic anaemias
(*c*) Haemolytic anaemias (*see* Chapter 9)
(*d*) Haemorrhagic anaemias (*see* Chapter 10)

After full investigation few anaemias are left in the first group but the classification cannot be regarded as complete since facets of one group may present in another A dyshaemopoietic anaemia for example may show evidence of haemolysis in excess of normal although by itself this may not be sufficient to produce any marked degree of anaemia

THE BLOOD COUNT

For screening purposes the estimation of haemoglobin must be accomplished by an examination of the stained blood film The appearance of hypochromia is no substitute for the mean corpuscular haemoglobin concentration (M C H C) but careful examination of the film might reveal evidence of an early red cell or white cell disorder which could be the cause of the anaemia

The absolute values classify the anaemias as macrocytic normocytic or microcytic and fully saturated or desaturated Since however the blood picture is merely a reflection of the activity of the blood forming tissues, the blood count in most cases must be regarded as indicating what other investigations may be necessary In this connexion the reticulocyte count is indispensable since it

truly reflects marrow activity The platelet count while not an obvious indication of anaemia is essential in those disorders in which there is depression of the haemopoietic processes

BONE MARROW EXAMINATION

The marrow may be obtained by either needle puncture or trephine biopsy The latter method provides more information in that the architecture of the marrow is preserved and cells are found in the biopsy specimen which are not dislodged by the suction process of needle puncture However, needle puncture is a simple procedure and in most cases provides adequate information Local anaes thesia renders the operation painless except during suction when a typical pain is experienced

Needle puncture

Choice of marrow puncture site—The choice of site for marrow puncture is determined by the age of the patient and degree of obesity In the child under 2 years of age the head of the tibia is a most suitable site since the bone is soft and usually well filled with marrow The iliac crest may be used in the patient aged up to about 12 years in the adult the sternum is the site of choice The sternum is close to the skin and marrow sites are located in the manubrium and opposite the second and third intercostal spaces In very fat individuals the spinous processes of lumbar vertebrae may be used This is also a good site in children since the patient is easily immobilized in a curled up position and the spinous pro cesses are prominent

Marrow puncture needles—A marrow puncture needle (Fig 19)

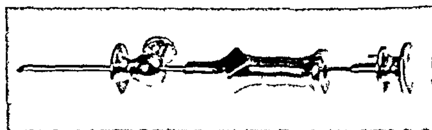


FIG 19—Salak marrow puncture needle (*Reproduced by courtesy of Charles Hearson & Co Ltd London*)

is a stout short bevelled needle with cutting edges equipped with a stilette and a guard The instrument may be driven into the marrow cavity by gentle taps with a hammer or preferably by rotary move

ments of a handle In either case the guard is so adjusted that deep penetration is impossible On entry into the marrow cavity the stilette is withdrawn and a dry syringe attached to the needle

Marrow smears—Only a small amount (0 25 ml) of marrow should be withdrawn to avoid dilution and contamination with blood Smears may be made directly on slides from the syringe nozzle or the material discharged into Sequestrene Sequestrene has an unfortunate effect in that plaques of malignant cells may be broken up thus possibly clouding the diagnosis Where malignancy is suspected it is recommended that a double oxalate mixture be used and smears made rapidly to avoid distortion If an anti coagulant is used actual marrow fragments may be picked out and smears prepared without squashing since this damages the cells Marrow smears must be dried by waving in the air immediately since thick slow dried smears show cell distortion Histological prepara tions may be made by allowing the marrow to fall into Helly s fluid and centrifuging subsequent treatment with dehydrating agents making the fragments easier to handle

Microscopic appearances—The fluid aspirate is sometimes counted as for a leucocyte count but since for accuracy this is dependent upon the degree of blood dilution the technique has limited value The stained preparation is first examined with the low power objective to obtain a general impression of the cellularity a hypoplastic or aplastic marrow specimen being immediately apparent Excess or absence of megakaryocytes is also easily seen these large cells usually being found in the tails of the film The higher power objective is needed for fine cytoplasmic and nuclear detail A differential count is performed on 500 cells to include all nucleated red cells and all leucocytes Note is made of mitoses and any cells which are not obvious blood cells or their precursors Some typical counts are shown in Table III From the counts two ratios may be calculated to indicate the relative activity of the red and white cell series The myeloid erythroid ratio (M E R) is the ratio of all myeloid cells to nucleated red cells and normally varies from 8 1 to 2 1 The leuco erythrogenic ratio (L E R) is the ratio of immature granulocytes to red cell precursors and varies from 1 5 1 to 2 5 1 Any white cells showing lobulation are not included in this ratio

Needle puncture marrow specimens taken from cadavers will show marked degeneration of the cells unless death is very recent The fragile cells may be protected from the trauma of spreading by collecting the marrow into a solution of ox albumen Smears made after this treatment yield good results

Trephine biopsy

Small trephines suitable for marrow biopsy are available including a very small type which is enclosed in a puncture needle The specimen is treated histologically, the interpretation of such sections (Plate I) needing considerable experience but in cases of replace ment by fibrosis or new growth the picture is typical Since the tissue is fixed and blocked many investigations may be made at leisure a proceeding not possible with needle puncture specimens

Other investigations

Bearing the definition of anaemia in mind it is obvious that investi gations relative to blood production and destruction must be carried out During haemoglobin synthesis porphyrin III is necessary, porphyrin I being produced as a by product This is excreted as coproporphyrin I in urine and faeces and erythropoietic activity may be estimated on the amounts excreted Urobilinogen excretion is affected by red cell destruction but it is also derived from other sources The estimation of urinary and faecal urobilinogen how ever serves as confirmatory evidence of red cell destruction in excess of normal

The presence of iron demonstrable by the Prussian blue reaction in the marrow is a normal finding The human red cell is capable of incorporating iron until the reticulocyte stage hence such sideroblasts are found in the normal marrow but are absent in iron deficiency states An increase in red cells containing iron is evidence of defective haemoglobin synthesis

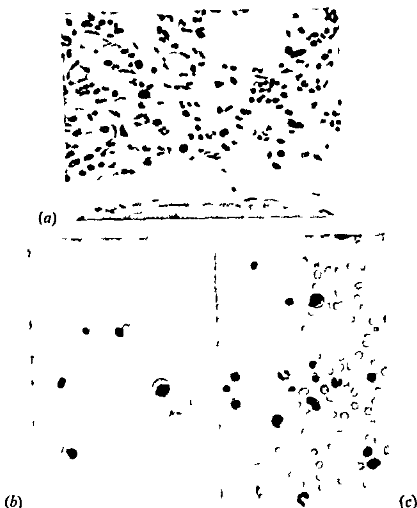
THE DYSHAEMOPOIETIC ANAEMIAS

Dyshaemopoietic anaemias arise from a deficiency of one or more of those factors which are necessary for the adequate production and proper maturation of the blood cells Such deficiencies may result from inadequate diet failure of gastric digestion or of intes tinal absorption inability to store or failure to utilize the ingested nutrients The manner in which such deficiencies arise is shown in Fig 20

Iron deficiency

The mechanism of iron absorption has already been discussed (Chapter 2) absorption occurring only in the upper small intestine where the reaction is favourable Achlorhydria may result in failure to absorb iron salts and consequent lack of response to oral therapy

PLATE I



(*a*) Iliac crest biopsy the marrow is replaced by a new tissue in which the only supporting structure appears to be proliferated capillaries Infiltration by plasma cells eosinophils megakaryocytes and groups of erythropoietic cells (*b*) Sternal marrow puncture megaloblastic reaction—pernicious anaemia (*c*) Sternal marrow puncture normoblastic reaction—haemolytic anaemia

(*To face page 74*)

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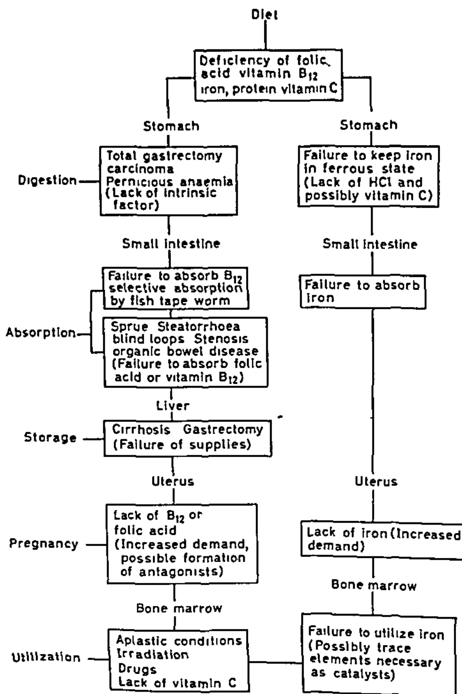


FIG 20.—The dyshaemopoietic anaemias

finely stippled nucleus with little tendency of the chromatin to clump and shows early haemoglobinization in the absence of associated iron deficiency

Classification of megaloblastic anaemias

The megaloblastic anaemias may be classified into three groups (as arranged by Witts) these are as follow

Primary deficiency of vitamin B_{12} or folic acid—This may be purely dietary the condition occurring in strict vegetarians but is not common The nutritional anaemias of the vitamin B_{1} folic acid iron and other essential factors, the condition being aggravated by chronic infection are those seen usually in under developed countries of the tropics

Relative deficiency of vitamin B_{1} and folic acid—Drugs which act as antagonists of these factors may cause a megaloblastic anaemia and pregnancy may produce the same condition possibly due to some abnormal product of metabolism In pregnancy faulty diet may also play a part

Conditioned deficiency of vitamin B_{12} or folic acid—This group includes addisonian pernicious anaemia and that due to intestinal disorders

Vitamin B_{12} deficiency

The haemopoietic factor vitamin B_{1} is a growth factor for various organisms It contains one cobalt atom in its molecule and is found in high grade protein foodstuffs It becomes bound to protein in gastric juice and serum the binding factor being inactivated at 100° C for 15 minutes

Castle s original theory of the aetiology of megaloblastic anaemias postulated that an extrinsic factor derived from food was combined in the stomach with an intrinsic factor elaborated by the gastric mucosa and was absorbed as haemopoietic principle The extrinsic factor is actually vitamin B_{1} the intrinsic factor is believed to be a mucoprotein The haemopoietic principle circulates in the serum as bound vitamin B_{12} but can be freed by boiling for 15 minutes Addisonian pernicious anaemia a disease of middle and old age is characterized by achlorhydria associated with a loss of gastric ferments and intrinsic factor Carcinoma of the stomach may produce a similar picture and total removal of the stomach results in the development of a megaloblastic anaemia as soon as the liver stores of B_{12} are depleted—a process which often requires a period of 2–4 years

The megaloblastic anaemia produced by infestation with the

The blood picture of iron deficiency depends on the duration of the deficiency, one of long standing producing a microcytic appearance The red cells, however, may be normocytic or even macrocytic hypochromia producing pessary and target cells Micropoikilocytes are common The mean corpuscular haemo globin concentration (M C H C) is reduced depending on the degree of iron deficiency The marrow shows a normoblastic reaction (Plate I) roughly proportional to the degree of anaemia the predominant cell being a small polychromatic normoblast with scanty cytoplasm and characteristic irregular cell outline The erythroblasts show late haemoglobinization and very little or no iron can be demonstrated by Perl s technique (*see* below)

Intravenous and intramuscular iron therapy by passes the mechanism of selective absorption making overdosage a real danger Deposition of iron in the pancreas and liver can cause fibrosis followed by diabetes and cirrhosis

THE MEGALOBLASTIC ANAEMIAS

Vitamin B_{12} and folic acid are materials which in some manner control the maturation of the red cell series Deficiency of one or both factors produces a characteristic anaemia The blood picture in most cases is of a fully saturated macrocytic anaemia in the absence of associated iron deficiency The mean corpuscular volume (M C V) may be as high as 150 cubic microns and anaemia may be severe Poikilocytosis and anisocytosis are marked and there is usually some leucopenia and thrombocytopenia Howell Jolly bodies Cabot rings (Frontispiece) and actual nucleated red cells may be seen in the peripheral blood The characteristic megalo blast seen in the blood is diagnostic The granulocytes are affected showing a right shift with the appearance of macropolycytes (Frontispiece)

The bone marrow shows a neoplastic type of proliferation of the erythroblasts (Plate I) Numerous mitoses are seen—cells larger than normal by reason of increased amount of chromatin and sometimes cytoplasm [1] increased number of early erythroblasts and all cells showing nuclear immaturity Desoxyribonucleic acid is deficient thus the process of division is held up even relatively mature megaloblasts tending to be Feulgen negative whereas even the pro erythroblast of the normoblast series is Feulgen positive The characteristic megaloblast is larger than the normoblast has a

[1] For a description of the mechanism by which polyploid cells are pro duced see Chapter 7

control the administration of such drugs since the use of 4 aminopteroyl glutamic acid and related compounds have resulted in the production of a true megaloblastic anaemia

The investigation of megaloblastic anaemia

I am indebted to Dr G I M Ross for permission to reproduce the following scheme of investigation Some of the techniques are not the province of the haematology department but are included for the sake of completeness

(1) Full blood count with absolute values and reticulocyte count
(2) Marrow puncture
(3) Serum B_{12} concentration (two separate samples since daily variations occur)
 80$\mu\mu$g /ml indicates severe B_{12} deficiency
 80-140$\mu\mu$g /ml uncertain must be repeated
 140$\mu\mu$g /ml excludes severe B_{12} deficiency
(4) Fractional test meal (preferably the augmented histamine meal of Kay) Free hydrochloric acid usually excludes addisonian pernicious anaemia
(5) Fat balance normal balance excludes idiopathic steatorrhoea
(6) Test if megaloblastic anaemia is due to deficiency of (*a*) vitamin B_{12} (*b*) folic acid (*c*) vitamin B_{12} + folic acid
 (i) Give 100$\mu\mu$g B_{12} intramuscularly (single dose only) Perform daily reticulocyte counts on fifth tenth fifteenth and et cetera days do haemoglobin estimation red-cell count and packed cell volume estimation (P C V) and calculate absolute values *Optimal response* marrow completely normoblastic on sixth day indicates pure vitamin B_{12} deficiency *Sub optimal* response indicates either pure folic acid deficiency or double deficiency of folic acid and vitamin B_{12}
 (ii) If response to vitamin B_{12} is sub optimal give 20 mg folic acid daily by mouth and perform daily reticulocyte counts and calculate absolute values on fifth tenth fifteenth and etcetera days
(7) Other investigations which may be of value in deciding aetiology of the anaemia are (*a*) glucose tolerance (*b*) iron absorption (*c*) vitamin A absorption (*d*) folic acid clearance test (*e*) folic acid absorption, (*f*) ascorbic acid absorption (*g*) uropepsin excretion (*h*) intrinsic factor activity of gastric juice, (*i*) gastric and intestinal biopsy (*j*) liver biopsy—vitamin B_{12} concentration

The use of radioisotopes —Radioactive vitamin B_1 is a useful tool in the investigation of megaloblastic anaemia Administered together with carbachol as a stimulant of intrinsic factor secretion the absorption of the radioactive B_{12} can be measured by the amount excreted in urine and faeces Liver and plasma radioactivity are

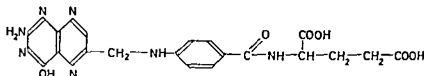
fish tape worm *Diphyllobothrium latum* is almost certainly due to the preferential demands for B_{12} made by the worm In a sense the tapeworm and the intestinal mucosa are competitors for the avail able B_{12} Other intestinal disorders such as sprue and steatorrhoea prevent absorption of haemopoietic principle and surgical abnor malities of the bowel such as stenosis and blind loops probably act by stagnation and putrefaction of the bowel contents thus destroying B_1 or breaking the bond which renders it capable of being absorbed

The liver must be badly damaged before it ceases to act as a storehouse for B_1 but cirrhosis can produce a megaloblastic anaemia for this reason

Vitamin B_{12} can be estimated in body fluids or tissue extracts by using the alga *Euglena gracilis* (var Z) as a test organism since it will detect as little as 0 25$\mu\mu$g /ml of B_{12} It is not completely specific responding to some pseudo B_1 s but these do not occur in serum and in any case are not B_1 active for mammals and birds The normal serum B_{12} level is 140–800$\mu\mu$g /ml [1]

Folic acid deficiency

Folic acid is present in spinach, mushrooms liver, kidney, yeast and the green leaves of many plants Synthetic folic acid is termed pteroylglutamic acid and has the following formula



Folic acid is a growth factor for *Lactobacillus casei* and *Strepto coccus faecalis* and these may be used as test organisms in its assay Megaloblastic anaemias due to folic acid deficiency may not appear so severe in marrow preparations as those due to B_1 deficiency and experience will sometimes enable the technologist to suggest the probable deficiency Folinic acid a related compound is converted to folic acid when given orally to a subject with normal gastric secretion

Folic acid antagonists

Certain drugs used in the treatment of leucocyte dyscrasias and also some barbiturates possess the property of interfering with folic acid metabolism in the body It would seem necessary to

[1] The serum B_{12} concentrations shown were obtained with *Euglena gracilis* by the method of Ross G I M (1952) *J clin Path* 5 250

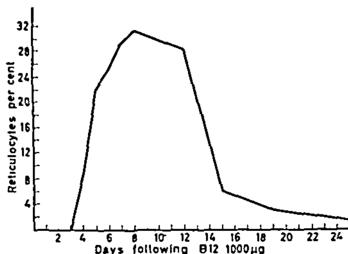


FIG 21 —Reticulocyte crisis, pernicious anaemia treated within vitamin B_{12}

POLYCYTHAEMIC STATES

DEFINITION

Polycythaemia is defined as a condition characterized by an increase in the number of red blood cells Polycythaemia may be relative, due to haemoconcentration from loss of fluid or compensatory, due to anoxaemia either by reason of a rarefied atmosphere or pul monary disease A chronic polycythaemic condition is found in congenital heart disease

Primary polycythaemia

The main feature of primary polycythaemia is an erythroid hyper plasia and in the later stages of the disease myeloid hyperplasia appears sometimes terminating in leukaemia The red cell count is usually between 7 and 12 millions per c mm The haemoglobin varies from 16 6 to 27 g per cent and the mean corpuscular volume 68–80 cubic microns The total blood volume and blood viscosity is increased and the spleen enlarged in polycythaemia vera rubra, but a similar disorder termed polycythaemia hypertonica (Gaisbock's disease) is characterized by high blood pressure and absence of splenomegaly

Secondary polycythaemia

Secondary polycythaemia produced by fluid loss is a relative excess of blood cells due to haemoconcentration The polycythaemia of this condition and also compensatory polycythaemia of pul

also indicators of the success of administration The techniques are discussed in Chapter 12

BONE MARROW DYSFUNCTION

Failure of the marrow to utilize available nutrients results in a hypoplastic or aplastic anaemia Such a condition results from the use of mitotic poisons of the benzol group, nitrogen mustards and from irradiation A pure red cell aplasia is a rarity but in association with thymic tumours has been described Usually leucocyte and megakaryocytic maturation is affected by toxic agents before red cell hypoplasia or aplasia appears The inhibitory effect of the spleen in hypersplenism is always quoted as causing the blood picture of aplastic anaemia with a normal marrow, the hormonal effect of the spleen preventing cells from passing into the circulation

The blood picture of aplastic and hypoplastic conditions varies according to the series mainly affected Haemoglobin and red cell count progressively decrease in true red cell aplasia but since it is exceedingly rare for the whole marrow to be affected reticulocytes may be found A peculiarity of the stained blood film seen sometimes is a diffuse pink background

DI GUGLIELMO SYNDROME

Erythremic myelosis, as the Di Guglielmo syndrome is more generally known is a truly neoplastic condition characterized by erythroblastic hyperplasia The process is not reversed by vitamin B_{12} or folic acid and the disease runs a short and fatal course During the early stages the marrow picture is normoblastic with a leucoblastic hypoplasia and reduction in megakaryocytes The blood contains numerous immature erythroblasts some being atypical normoblasts and immature leucocytes appear as a terminal phenomenon The serum B_{12} level in this condition is unusually high

MACROCYTIC ANAEMIAS

Anaemias characterized by a macrocytic blood picture with a *normoblastic marrow occur in a variety of conditions Such an* anaemia sometimes occurs in myxoedema although microcytic or normocytic anaemias are more common The aetiology of the anaemia is obscure but primarily appears to be due to a depression of the erythron Macrocytic anaemias in pregnancy and malnutrition have the same basis of inadequate diet or increased demands The successful treatment of anaemias with low red-cell counts is shown by the appearance of a reticulocyte crisis (Fig 21)

(*c*) Staining reagent Mix equal parts of (*a*) and (*b*) immediately before use
(*d*) Counterstain 0 1 per cent safranin

Method

(1) Fix air-dried films of blood or marrow for 20 minutes in methanol
(2) Place slides in staining reagent in water bath at 55 C for 10 minutes
(3) Wash slides in running tap water for 20 minutes
(4) Rinse in distilled water
(5) Counterstain for 15 seconds with safranin

Siderotic granules stain as blue dots 1 5μ and smaller

SERUM IRON (MARRACK, D , Personal communication)

Reagents

The reagents used in the estimation of serum are

(*a*) Trichloracetic acid 60 per cent (w/v) trichloracetic acid in water
(*b*) Alkaline acetate solution N/1 sodium hydroxide saturated with solid sodium acetate (25 g /100 ml) Keep the solution in a tall bottle when needed remove portions for use without disturbing the deposit
(*c*) Ascorbic acid solution dissolve a knife point of ascorbic acid (0 25 g) in 3 ml of N hydrochloric acid Make the solution freshly each day
(*d*) Dipyridyl reagent dissolve a small knife point of ascorbic acid (0 1 g) and 10–15 crystals (0 01 g) of AR 2 2 dipyridyl in 2 ml of distilled water Make the solution freshly each day
(*e*) Iron standards—stock standard 0 862 g ferric ammonium sul phate ($7H_2O$) with about 100 ml concentrated HCl made up to one litre This contains 100μg /ml Standards containing 100 and 300μg /100 ml are obtained by further dilution with 0 01N HCl

Method

(1) Add 0 2 ml of ascorbic acid solution to 4 ml serum in a centrifuge tube After mixing by gentle shaking and allowing to stand for 5 minutes add 0 4 ml of trichloracetic acid solution and stir the mixture well for at least 45 seconds with a thin glass rod After standing for a further 10 minutes centrifuge for 20 minutes
(2) Place 0 4 ml of alkaline acetate solution and 6 drops of dipyridyl reagent in a test tube Add 2 ml of supernatant mix the contents and allow to stand for 10 minutes before reading in the spectro photometer at 520 mμ
(3) Treat 4 ml of distilled water and 4 ml of the standards in the same way as serum for blank and standards

monary disease is differentiated on the marrow picture from that of primary erythrocytosis In the latter condition all marrow elements are hyperplastic but except in altitude sickness, where there may be a normoblastic reaction the secondary polycythaemias have a normal marrow picture

TABLE IV
AVERAGE CELL PERCENTAGES IN MARROW OF DYSHAEMOPOIETIC ANAEMIAS

	Normal	*Pernicious anaemia*	*Iron deficiency*	*Aplastic anaemia*
Reticulum cell	0–1	1–3	0–1	0–1
Haemocytoblast	0–1	0–3	0–1	0–1
Pro-erythroblast	0–2	1–3	1–3	0–1
Early normoblast	1–3	1–5	1–10	0–1
Intermediate normoblast	5–15	5–15	15–35	2–6
Late normoblast	1–8	5–10	1–10	1–4
Pro-megaloblast	0	3–10	0	0
Early megaloblast	0	2–15	0	0
Intermediate megaloblast	0	4–25	0	0
Late megaloblast	0	3–10	0	0
Red-cell mitoses	0–1	1–2	0	0
Myeloblast	1–4	1–4	1–4	0–1
Pro myelocyte	1–10	1–12	1–5	0–3
Neutrophil myelocyte	2–20	12–20	2–10	1–7
Eosinophil myelocyte	1–3	1–2	0–1	0–1
Basophil myelocyte	0–1	0–1	0–1	0
Metamyelocyte	10–48	5–20	5–15	4–15
Neutrophil polymorphonuclear	9–34	3–12	5–15	5–10
Eosinophil polymorphonuclear	1–3	1–2	0–1	0–1
Basophil polymorphonuclear	0–1	0–1	0–1	0
Lymphocyte	2–20	6–12	2–15	30–50
Monocyte	0–5	0–2	0–3	3–8
Plasma cell	0–2	0	0	0
Megakaryocyte	0–2	0	0	0
Myeloid erythroid ratio	8 1–2 1	2 1–1 1	4 1–1 1	8 1–1 1
Leuco-erythrogenic ratio	1 5 1–2 5 1	0 3 1–1 6 1	0 5 1–1 5 1	1 1–2 5 1
Comment		Megalo blastic reaction	Normo blastic reaction	Hypo-cellular marrow

TECHNIQUE

STAINING OF SIDEROTIC GRANULES

Reagents

The reagents used in staining siderotic granules are

(*a*) Potassium ferrocyanide 2 per cent aqueous solution
(*b*) N/5 hydrochloric acid

Metals 45 dry mix

Ferrous ammonium sulphate ($FeSO_4(NH_4)_2SO_4 6H_2O$)	14 0 g
Zinc sulphate ($ZnSO_4 7H_2O$)	4 4 g
Manganese sulphate ($MnSO_4 H_2O$)	1 54 g
Copper sulphate ($CuSO_4 5H_2O$)	0 32 g
Cobalt sulphate ($CoSO_4 7H_2O$)	0 48 g
Boric acid (H_3BO_3)	0 57 g
Ammonium molybdate ($(NH_4)_6Mo_7O_{24}4H_2O$)	0 64 g
Sodium orthovanadate ($Na_3VO_4 16H_2O$)	0 0925 g
	22 04 g for 1 000 litres of final medium

Grind the ingredients together and store in a dry place in a polythene bottle

Stock B_{12} solutions (freshly prepared every three weeks)
Ampoule of B_{12} (1 ml) contains 20μg = 20 000$\mu\mu$g

Bottle No	*Final conc* $\mu\mu$g /ml	*Initial conc*	*Dilution required*	*Ml of initial conc*	*Ml of aqua dist*
1	200 000	20 000 000	1 100	1 0	99
2	2 000	200 000	1 100	1 0	99
2*a*	100	2 000	1 20	5 0	95
3	20	2 000	1 100	1 0	99
3*a*	2	20	1 10	10 0	90

The solutions must be prepared using sterile glassware the final products being regarded as sterile

Stock culture

Maintain stock cultures in Pyrex 6 inch × ¾ inch tubes of tryptone in agar

Apparatus

All glassware must be neutralized by filling with distilled water and autoclaving at 5 pounds for 30 minutes then dried plugged and sterilized in the oven at 160° C for one hour

To set up a batch of serum

(1) Set up each test serum in three dilutions 1 20 1 40 and 1 80 in duplicate
(2) Steam these tubes together with the standards in triplicate sera of known B_{12} value diluted as for tests in triplicate and two tubes of 1 20 dilution of a known low serum for 15 minutes to free any combined B_{12} This gives the total B_{12} present

Calculation

Low standard $\frac{\text{Reading of test}}{\text{Reading of standard}} \times 100$ = serum iron (μg /100 ml)

High standard $\frac{\text{Reading of test}}{\text{Reading of standard}} \times 200$ = serum iron (μg /100 ml)

The standard with the reading closest to that of the unknown is used

Note All apparatus bottles and syringes should be boiled in dilute hydrochloric acid and washed in distilled water

ASSAY OF VITAMIN B_{12} (ROSS)

Media

Media to be used in the assay of vitamin B_{12} are as follow

Stock culture medium Tryptone (Oxo Ltd) 0 2 per cent in 0 25 per cent agar This medium contains the equivalent of 20$\mu\mu$g /ml of B_{12}

Stock culture medium 50 + T Double strength basal medium 5 0 ml Tryptone 0 4 per cent 4 75 ml B_{12} 2 000$\mu\mu$g /ml 0 25 ml of bottle 2 (*see* page 85) This medium contains 50$\mu\mu$g /ml of B_{12} and is used for cultures from which inoculums will be made into medium + test fluid

Double strength basal medium	*Concentration mgm per cent in final medium*	*Weight (g) for 10 litres final medium*
Potassium dihydrogen phosphate (KH_2PO_4)	30	3 0
Magnesium sulphate ($MgSO_4 7H_2O$)	26	2 6
Magnesium carbonate ($MgCo_3$)	14	1 4
L glutamic acid	300	30 0
Calcium carbonate ($CaCO_3$)	8	0 8
Sucrose	1 500	150
DL-Malic acid	100	10
DL-aspartic acid	200	20
Glycine	250	25
Ammonium succinate ($(CH_2 COO NH_4)_2$)	60	0 6
[1]Thiamine HCl	0 06	0 6
Metals 45	2 2	0 22
Para amino benzoic acid	0 125	0 013

[1] This is 0 6 triturate containing 1 g of thiamine hydrochloride + 99 g sucrose (1 100 triturate) Dissolve the ingredients in 5 000 ml distilled water and steam for 15 minutes *Cool and adjust pH to 3 6 with 40 per cent NaOH* or 40 per cent M_2SO_4 Filter bottle and sterilize by autoclaving at 10 pounds for 15 minutes

Reading of results

Plot the standard curve for B_{12} on 3-cycle semi log paper Plot the B_{12} concentration on the logarithmic side and the turbidity reading on the linear side Corrections may be made for turbid or coloured test fluids first by reading the turbidity and then sedimentation of the alga (immobilized by caprylic alcohol) or after centrifugation reading the turbidity of the supernate

This value is subtracted from the total turbidity

Read concentration of test cultures from the standard curve and multiply by the dilution to obtain values of $\mu\mu$g /ml serum

Normal values 140–800$\mu\mu$g /ml

PRESERVATION OF MARROW CELLS

Marrow cells are preserved as follows

(1) Withdraw 0 25 ml of marrow and discharge into five times its volume of 10 per cent ox albumen
(2) Centrifuge lightly and make smears from the deposited marrow fragments

QUANTITATIVE ESTIMATION OF URINARY UROBILINOGEN (WATSON)

Reagents

The reagents used in quantitative estimation of urinary urobilinogen are as follow

(*a*) Ehrlich s reagent (modified)

> Paradimethylaminobenzaldehyde 0 7 g
> Concentrated hydrochloric acid 150 ml
> Distilled water 100 ml

(*b*) Saturated aqueous pure sodium acetate
(*c*) Stock standard

> Pontacyl Carmine 2B 5 mg
> Pontacyl Violet 6R 150 per cent 95 mg
> Acetic acid 0 5 per cent 1 000 ml

(*d*) Working standards

> Stock standard sol 10 ml
> Acetic acid 0 5 per cent 50 ml } Colour intensity equivalent to 0 6 mg per cent urobilinogen

Method

(1) Empty patient s bladder in early afternoon
(2) Give patient half a pint of water to drink
(3) Collect sample 2 hours later measure volume and cool to room temperature

(3) Heat two tubes of 1 40 dilution of each test serum at 51° C for 30 minutes This gives the free B_{12}

(4) Steam two tub s of 10 ml amounts of stock medium and 20 ml and 10 ml of single strength basal medium for 15 minutes

The Test Proper

TABLE V

TO SET UP A RANGE OF STANDARDS

No	*Final conc* $\mu\mu g$ */ml*	*Init* B_{12} *bottle*	*Ml of init* B_{12}	*Ml of D* H_2O	*Basal medium D S*
1	50	2*a*	2 0	—	2 0
2	25	2*a*	1 0	1 0	2 0
3	15	2*a*	0 6	1 4	2 0
4	10	2*a*	0 4	1 6	2·0
4*a*	10	3	2 0	0	2 0
5	5	3	1 0	1 0	2 0
6	2 5	3	0 5	1 5	2 0
7	1 25	3	0 25	1 75	2 0
8	1 0	3*a*	2 0	—	2 0
9	0 5	3*a*	1 0	1 0	2 0
10	0 25	3*a*	0 5	1 5	2 0
CT	0	—	—	2 0	2 0

Thus all tubes are made up to a final volume of 4 ml For routine purposes set up the standards CT 9 7 6 5 4 4*a* 3 2 1 in triplicate

Prepare fresh stock solutions every 3 weeks and set up tubes 2 5 9 of the old stock against the new standards for comparison

Preparation of inoculums

(1) Select a stock culture preferably 5–6 days old and check the turbidity for contamination

(2) Spin the cells at 2 000 r p m for 5 minutes decant the supernatant and resuspend the cells in 20 ml single strength basal medium mix thoroughly respin decant and finally resuspend in 10 ml single strength medium This washes the cells free from any binding material produced by the Euglena (*see* above)

(3) Inoculate the cooled tubes with one drop of this suspension from a Pasteur pipette held vertically to control the size of the drop

(4) Incubate the tubes at 28 C (optimum temperature) for 5 days and read their turbidity on the sixth day using a quarter cell in the photo electric colorimeter and using a spectral red Ilford filter No 608 The temperature should not be allowed to fall below 26 C or rise above 32° C

Note Vigorous shaking is required to disperse clumps of algae and tubes with serum froth markedly Bubbles are dispersed immediately by adding one drop of caprylic alcohol water (1 in 5) This should not be added before shaking because of the fragmentation of algae and cloudiness which may result

(9) After mixing read in colorimeter using Ilford spectrum yellow green filter No 605 against a calibrated neutral grey screen or a phenolphthalein standard

Calculation

Mgm urobilinogen per 100 g stool $= \frac{3\,87 \times U \times V}{S}$ where U is reading of unknown V is final volume of coloured solution in ml and S is reading of standard

Note A blank of 2 ml filtrate plus 2 ml 6N hydrochloric acid and 6 ml of sodium acetate solution diluted in the same volume as the test is used as a blank for zero setting of the colorimeter

(4) Place 2 5 ml in colorimeter tube and add 2 5 ml Ehrlich reagent and 5 0 ml saturated aqueous pure sodium citrate
(5) Mix thoroughly and read in colorimeter using an Ilford spectrum yellow green filter No 605

Calculation urobilinogen concentration plus allied substances is calculated from

$$\frac{\text{Urobilinogen in mg per 100 ml}}{100} \times \text{dilution} \times \text{volume of 2 hour sample} = \text{No of Ehrlich units in sample}$$

The normal is less than 1 unit

QUANTITATIVE ESTIMATION OF FAECAL UROBILINOGEN (MACLAGAN)

Reagents

The reagents used in the quantitative estimation of faecal urobilinogen are as follow

(*a*) Ferrous sulphate 20 per cent $FeSO_4 7H_2O$ 20 g distilled water 92 ml
(*b*) NaOH 10 per cent
(*c*) Modified Ehrlich reagent (Watson)
(*d*) Saturated aqueous pure sodium acetate solution
(*e*) 6N hydrochloric acid
(*f*) Phenolphthalein standard

(1) Dissolve 50 mg phenolphthalein in 100 ml absolute ethanol
(2) Add 1 ml of this solution to 10 ml 10 per cent sodium carbonate (Na_2CO_3) solution and dilute to 100 ml with water immediately before comparison

The standard is equivalent to 0 387 mg urobilinogen per 100 ml

Method

(1) Weigh out 1 5 g of fresh formed stool and transfer to a 6-inch × 1 inch test tube
(2) Add 9 ml of distilled water and mix thoroughly
(3) Add 10 ml of ferrous sulphate solution and mix well
(4) Add 10 ml of sodium hydroxide solution and mix well
(5) Allow to stand 1–3 hours
(6) Filter through Whatman No 1 11 cm paper
(7) Rapidly place 2 ml of filtrate in a dry 250 ml measuring cylinder and add 2 ml of Ehrlich reagent Mix and allow to stand 10 minutes
(8) Add 6 ml of saturated sodium acetate solution shake and then dilute with distilled water according to colour which develops

somes remain whole, and in the spermatocyte one of each chromosome pair proceeds to opposing ends of the cell, so that the diploid number of chromosomes is reduced to the haploid number in the mature spermatozoon During the maturation of the ovum the chromosomes separate and form two nuclei one of which is eccentric

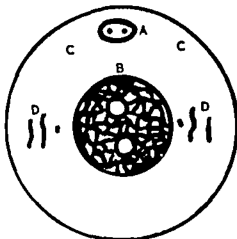


FIG 22 —The cell A Centrosome with centrioles B nucleus with nucleoli C cytoplasmic inclusions (yolk fat glycogen mucus and so on D mitochondria



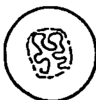










FIG 23 —The cell in mitosis 1 and 2 prophase 3 metaphase 4 and 5 anaphase 6 telephase

CHAPTER 7

THE CELL AND INHERITANCL

THE CELL

COMPOSITION OF THE CELL

THE CELL (Fig 22) consists of cytoplasm and its inclusions nucleus and possibly nucleoli The cytoplasmic inclusions are centrioles inside the centrosome mitochondria and inclusions related to the function of the differentiated cell Yolk fat, glycogen mucus and so on are such specific inclusions

CELL DIVISION

The normal means of growth and replacement of cell wastage in the body is by mitotic division (Fig 23) of the cells The first stage of *mitosis* is termed *prophase* and is characterized by the centrioles moving apart and the appearance of attraction rays radiating from the centrosome At the same time the nucleus becomes a long thread of material termed the spireme This stage merges into *metaphase* in which the centrioles are at opposing ends of the cell and the astral lines radiate everywhere being particularly numerous in the zone between the centrioles where they form the spindle The chromatin thread meanwhile has broken into small pieces called chromosomes which lie suspended along the spindle in one plane called the equatorial plate The chromosomes split longitudinally and in the next stage one of each pair so produced moves to opposing ends of the cell and at the same time each centriole divides in prepa ration for future division this is termed *anaphase* In the last stage the chromosomes at each end of the cell fuse to form the spireme nuclear membranes are assumed and the astral lines begin to fade A constriction which has appeared in the cell becomes marked and the two cells begin to part this stage is termed *telophase* Finally the nuclei having been fully restored to the shape in the resting cell the two daughter cells separate and after an interval the process is repeated

The number of chromosomes in a cell is constant for a given species but the sex cells mature in a manner which reduces this number by one half Instead of splitting at metaphase the chromo

GENES

To explain the appearance of only one characteristic when two genes are operating it is necessary to postulate that one gene has the power to blot out the effect of another This power is termed dominance, and a gene which is capable of exerting such influence is called a dominant gene The gene which is submerged is said to be recessive and such genes in some cases are bad ones in that a double dose may produce unfortunate characteristics Inbreeding often brings bad characteristics to the surface since the chances of a double dose of bad recessive genes being inherited are greatly increased Careful selection of matings, however even in closely related communities can produce only good results

Lethal genes

Recessive characteristics inherited in a double dose sometimes cause the death of the infant *in utero* and the genes are termed lethal genes It is believed that the gene of haemophilia may sometimes act in this way

Modifying genes

It has been known for a long time that the genes for one characteristic may have the effect of modifying or suppressing the action of another and in recent years such interference with gene expression has been demonstrated in human beings It is not known how modifying genes exert their effect but examples are found in the inheritance of blood groups

Linkage

For the inheritance of a single factor one site on the chromosome is available Should there be more than one version of this characteristic there will be other genes for the factor which are regarded as competitors for the chromosomal site Such alternative genes are termed allelomorphs or alleles Genes which occupy sites very close to one another on the chromosome so that they are always inherited together are said to be linked

Crossing over

The chromosomes at one stage during meiosis are so close together that they tend to adhere When they separate again it occasionally happens that the semi fluid material has fused so that a piece of chromatin belonging to one chromosome has become an integral part of the second and vice versa (Fig 24) In such cases a

and is termed a polar body This is extruded from the cell so that the mature ovum only contains the haploid number of chromosomes This type of division is termed *meiosis*, amitotic or reduction division To obtain a full complement of chromosomes it is necessary that the ovum be impregnated by the sperm and the two pronuclei fuse Reproduction then proceeds by normal mitotic division

Most authorities agree that cells of the haemopoietic system are normally produced by mitotic division from a totipotential pre cursor but the megakaryocytes then mature by a process known as *endomitosis* in which no obvious division takes place the nucleus and cytoplasm merely doubling in size Under pathological con ditions the blood cells divide in a manner which produces polyploid cells—cells containing more than the normal number of chromo somes A polyploid cell may contain a single large nucleus several diploid or polyploid nuclei or a mixture of both types Twinning deformities in which the cell contains two nuclei which are mirror images of one another are produced by the division of the nucleus without division of the cytoplasm Small blood cells with less than the normal number of chromosomes are seen sometimes in blood diseases The micromyeloblast is not regarded as such a cell it being merely deficient in cytoplasm The exact number of chromo somes cannot be determined in blood cells polyploidy being usually characterized by the size of the cell or nucleus or both Giant chromosomes are sometimes seen in normoblasts and megaloblasts in the anaemias

INHERITANCE

THE STRUCTURE OF THE CHROMOSOMES

In human cells there are 23 pairs of chromosomes of which 22 are twins but the last pair do not resemble one another in males while in females they are identical and moreover, resemble one of the odd pair in the male The chromosomes attach themselves to the spindle by a small particle called the centromere at which point they begin to split On the chromosomes themselves are numerous small beads or bands which although constant in structure and number for a species differ from one another These are termed chromomeres and are believed to be the site of the factors responsible for the inheritance of physical and some mental characteristics The hypothetical determining centres are called genes and since they are carried on chromosomes donated from both parents it follows that the infant will inherit a mixture of characteristics

and other terms are illustrated by the experiments of Mendel with green and yellow seeded garden peas

In Family (1) (Fig 25) yellow seeded peas were crossed with green seeded peas and a yield of 100 per cent yellow seeded peas obtained Multiplying the genotype constituents together shows that from two homozygotes 100 per cent of heterozygotes is obtained, and in the family illustrated that yellow is dominant over green When the heterozygotes were crossed with each other Mendel obtained yellow to green seeded peas in a ratio of 3 1 From Fig 25 (*b*), it will be seen that yellow is dominant since green only appears in double dose

Family 1

	Y	Y
g	Yg	Yg
g	Yg	Yg

(a)

Family 2

	Y	g
Y	YY	Yg
g	Yg	gg

(b)

FIG 25 —The Mendelian experiments with green and yellow seeded garden peas families 1 and 2 illustrated diagrammatically

A definition of a Mendelian dominant therefore is a gene which expresses itself in single or double dose and a Mendelian recessive is a gene which expresses itself only in double dose

CALCULATIONS

(1) The number of possible chromosomes for allelomorphic genes is equal to the number of allelomorphs

(2) The possible number of chromosomes with paired genes can be calculated using the square rule in which the number of paired genes is multiplied together for example, 4 pairs of genes $2 \times 2 \times 2 \times 2 = 16$ possible chromosomes

(3) The number of possible genotypes when dealing with the inheritance of a single factor is obtained from the formula $\frac{n}{2}(n + 1)$ where n is the number of chromosomes For example, for the blood group factor M and its allelomorph N there are two possible chromosomes The possible genotypes are obtained from $\frac{2}{2}(2 + 1) = 3$ and on inspection these are MM, MN and NN

rarer chromosome may be produced Crossing over does not separate some genes and in such cases the linkage is said to be very close

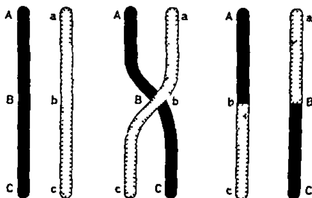


Fig 24—*Diagram illustrating crossing over* The chromosomes ABC and abc produce Abc and aBC

SEX LINKED CHARACTERISTICS

Sex is determined by the possession of the odd chromosome in the normal set The human male possesses one dissimilar pair of chromosomes designated X and Y the X chromosome being identical with another pair in the female During the formation of the gametes one sperm will receive the Y and the other the X chromosome An ovum fertilized by a sperm carrying the Y chromosome will result in a male infant and that fertilized by a sperm carrying the X chromosome will result in a female infant The X and Y chromosomes carry other genes and since the effect can only be exerted in the sex predetermined by the gamete such genes are said to be sex linked

MENDELISM

The genetic make up of an individual as determined by observation laboratory experiment and pedigree studies is known as the genotype or type which creates It is the determinant whereas the effect produced and observable is the phenotype or type which is seen The phenotype is defined as the genetic make up of an individual as determined by observation and limited laboratory experiment Rarely the genotype can be assumed from the phenotype

An individual who has inherited identical genes for a characteristic from both parents is said to be homozygous and an individual who has inherited dissimilar genes is said to be heterozygous These

The haemolytic anaemias however, especially hereditary sphero cytosis are characterized by aplastic crises in which the tired marrow temporarily ceases to function Crises of this nature are usually associated with infections

INTRAVASCULAR HAEMOLYSIS

Two rates of haemolysis are considered here

(*a*) The chronic small or slow haemolytic process and (*b*) the acute or rapid destruction of a large number of cells

The two types may be conveniently compared with the normal destruction of effete red cells, by tabulating the increase of products of haemolysis and by products of haemoglobin synthesis (Fig 26)

Methaemoglobin may appear in plasma and urine following intravascular haemolysis, but only if present in the destroyed red cells Methaemalbumen a compound of haematin and serum albumen appears in the plasma shortly after a haemolytic episode and is detectable spectroscopically up to 48 hours afterwards The molecule is too large to pass the kidney and therefore it does not appear in the urine Carbonic anhydrase, an enzyme present in red cells is concerned with the liberation of CO_2 from bicarbonate In rapid haemolysis the enzyme is freed into the plasma and may be excreted in the urine but its demonstration is only of academic interest Haemosiderin the iron containing pigment of haemoglobin breakdown is always found in the urine of chronic haemolytic anaemia, but a single haemolytic episode however intense may not show the characteristic granules immediately since the kidney has first to absorb and then re excrete the pigment

Serum bilirubin level

The serum bilirubin level depends to a great extent on the efficiency of the reticulo endothelial system, hence it may in some cases be a misleading measure of haemolysis Excess urinary urobilinogen also may be an indication that the liver is unable to re excrete urobilinogen reabsorbed from the bowel rather than a sign of increased haemolysis The amount of faecal urobilinogen excreted is not a good measure of red cell destruction since as much as 20 per cent of faecal urobilinogen is derived from sources other than haemoglobin breakdown

Excretion of coproporphyrin

Coproporphyrin I is the form in which porphyrin I the by product of haemoglobin synthesis, is excreted and in haemolytic anaemia it is increased in amount Coproporphyrin III, however is only

CHAPTER 8

THE HAEMOLYTIC ANAEMIAS

GENERAL DATA

INTRODUCTION

THE HAEMOLYTIC ANAEMIAS are characterized by a reduction in the life span of the red cells which are destroyed in the blood vessels even when they are only a few days old The haemolytic process may be either an inherited or an acquired condition some of the inherited disorders having definite racial associations

An inherited haemolytic anaemia is one in which a familial tendency can be demonstrated, and is associated with anomalies of red cell shape and abnormal structure of haemoglobin Acquired haemolytic anaemias may be due to drugs poisons virus infection and allergy etc or to the formation of antibodies active against red cells Red cell shape may be altered in the acquired haemolytic anaemias

BLOOD PICTURE

Providing the marrow is functioning normally the blood picture is one of regeneration the degree of anaemia found being related to the time interval between haemolytic episode and blood examination The stained film shows anisocytosis with large macrocytes present polychromasia and basophilic stippling Fragmented cells known as schistocytes are usually present and target cells and sphero cytes despite their association with specific disorders may be found in any haemolytic anaemia Howell Jolly bodies and even normoblasts may be present in the peripheral blood and there may be some degree of leucocytosis due to an increase in the myeloid elements Reticulocytosis is invariable even where there is no obvious anaemia up to 50 per cent of reticulocytes being found in hereditary spherocytosis with a normal red cell count

Marrow response

The blood picture results from the normal marrow response to red cell destruction the marrow being hyperplastic and showing a normoblastic reaction Macro normoblasts may be prominent

excreted in excess where there is interference with haemoglobin synthesis by poisons, such interference being evident in haemolytic anaemias due to lead, arsenic, sulphonamides and other drugs The presence of siderocytes is another indication of interference with haemoglobin synthesis and they may be found in haemolytic anaemias, but they also occur in other blood disorders and after splenectomy

THE NORMAL RED CELL SHAPE

The normal red cell shape is a biconcave disc, a shape which presents the largest possible surface area to the plasma This is necessary because of the complex physical and chemical reactions which take place between red cell and plasma The shape also lends itself readily to the distortion necessary for it to pass the finer capillaries and would appear to be best able to withstand the inevitable trauma sustained in the blood stream The biconcave disc is also able to withstand limited changes of osmotic pressure without rupture

DEFECTS OF RED CELL SHAPE

Anomalies of red cell shape are inherited as Mendelian dominants, and are associated with the inheritance of abnormal haemoglobins (*see* below) or persistence of foetal haemoglobin into adult life

Spherocytosis

Hereditary spherocytosis is associated with a chronic haemolytic anaemia, the abnormal cells having a shortened life span Small amounts of foetal haemoglobin may sometimes be demonstrated in the adult blood The spherocytes are removed from the blood stream by the spleen removal of the organ being curative although spherocytosis persists The cells appear as deeply staining microcytes (Fig 27), the average diameter being 6 5–7 0 microns The M C V is usually normal or slightly decreased the volume being made up by the increased thickness The normoblasts and reticulocytes are of normal shape only the mature cells being spherocytic The marrow is hyperplastic except during aplastic crisis and there is usually some anaemia with a constant reticulocytosis The serum bilirubin rarely rises above 4 0 mg per cent The osmotic fragility (*see* below) of spherocytes is increased and even in mild cases the defect can sometimes be recognized by incubating the blood at 37° C for 24 hours when the osmotic fragility may be increased beyond that of a normal incubated specimen The spherocytic cell envelope also seems to be defective since incubation of sterile blood

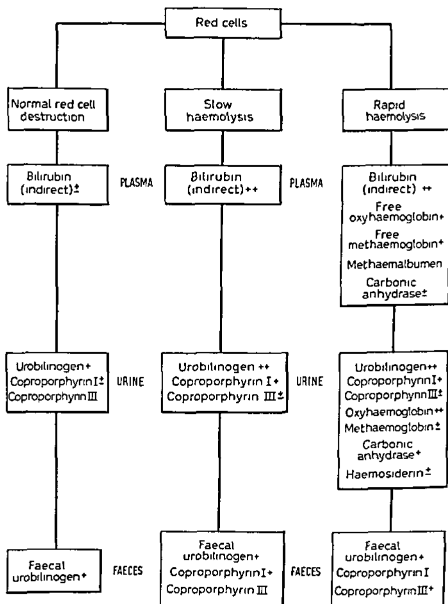


FIG 26—Comparison of slow and rapid haemolysis with normal physiological destruction of effete red cells

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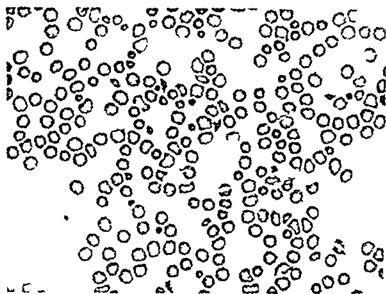


FIG 27 —Peripheral blood in hereditary spherocytosis (×420)

results in spontaneous lysis (autohaemolysis of Dacie) in excess of similarly treated normal blood The abnormal cell also does not stand up to wear and tear, its mechanical fragility (*see* below) being markedly increased

Spherocytes are found in many haemolytic anaemias and there seems little doubt that poisons malignant and unknown processes can so modify the red cell that it becomes spherocytic in the absence of genetic influence An acquired spherocytosis in some cases seems to be associated with an increased glucose metabolism failure to provide the metabolite resulting in haemolysis *in vitro*

Elliptocytosis

Elliptocytosis is not always associated with a frank haemolytic anaemia and the defect does not make the cells more sensitive to changes in osmotic pressure Sometimes the cells are oval rather than elliptical and occasionally spherocytes may be seen although the genes governing the inheritance of spherocytosis and elliptocytosis are not related Some authorities regard elliptocytosis as occurring in three grades of severity haemolytic anaemia being the least common A less active type shows a reticulocytosis but is compensated in that there is no obvious clinical evidence of haemolysis In the latent type which is the most common elliptocytes are present but there is no evidence of haemolysis or anaemia The

immature cells in this condition are not elliptical, assuming this shape after they have lost their reticulum

Leptocytes

The leptocyte of Mediterranean anaemia (Fig 28) is an abnormally thin cell inherited as a Mendelian dominant and associated with this shape is a varying amount of foetal haemoglobin persisting into adult life The gene expresses itself in three grades of severity, termed thalassaemia major, minor and minima Thalassaemia major is found in the homozygote as a frank haemolytic anaemia while the heterozygote presents either as a mild haemolytic anaemia or is symptomless Inheritance of the trait, however, combined with another red-cell defect or an abnormal haemoglobin results in severe symptoms The leptocyte is resistant to changes in osmotic pressure, the cell remaining intact in very low concentrations of sodium chloride Due to its increased mechanical fragility the leptocyte has a reduced life span The characteristic cell of thalassaemia is the target cell, but this appearance is also seen in some iron-deficiency states In such cases the iron deficient leptocyte behaves in respect of osmotic and mechanical fragility as the cell of thalassaemia and it may be difficult without pedigree studies to distinguish between them It should be noted that in all cases of thalassaemia there is a high serum iron, in spite of the hypochromic red cell, suggesting a fault in utilization of iron

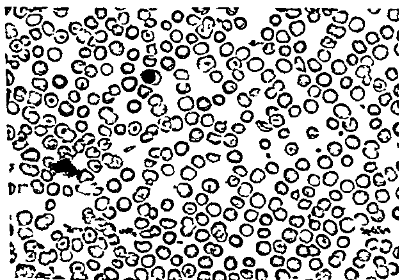


FIG 28 —Thalassaemia major in a Greek child (× 420)

Drepanocytes

Sickle cells or drepanocytes (Fig 29) are cells which under conditions of reduced oxygen tension assume bizarre shapes due to crystallization of an abnormal haemoglobin The sickling process is mainly reversible The anomaly is inherited as a Mendelian dominant and the amount of sickle haemoglobin is increased in the heterozygote with consequent severe symptoms if a gene for another abnormal haemoglobin or the thalassaemia gene is also inherited The heterozygote is usually symptomless but the combination of

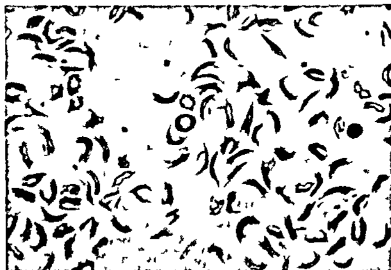


FIG 29—Sickle-cell anaemia in an African Negro the preparation was made from blood treated with a reducing agent (× 450)

sickle haemoglobin with the thalassaemia gene is not uncommon in some parts of the world the disorder being known as micro drepanocytic disease The sickle cell has a reduced life span and increased mechanical fragility but it is very resistant to changes in osmotic pressure

A DEFECT OF UNKNOWN NATURE

Paroxysmal nocturnal haemoglobinuria is a haemolytic anaemia due to the red cells becoming sensitized to and lysed by a component of normal serum Properdin the bacteriocidal globulin is involved in the haemolytic process the exact nature of which is unknown The reaction occurs *in vitro* at *p*H 7 0 that is slightly on the acid side of physiological *p*H is enhanced by thrombin and *in vivo*

occurs during sleep The osmotic and mechanical fragilities are normal Haemosiderinuria is a constant finding and there is usually a leukopenia due to a reduction in the myeloid elements and some thrombocytopenia The serum bilirubin is rarely in excess of 3 0 mg per cent The condition is diagnosed by the acid serum test in which the suspected cells are exposed to acidified serum with resultant haemolysis Blood showing marked spherocytosis also haemolyses but can be distinguished by repeating the test with inactivated serum P N H cells are not lysed by acidified heated serum.

OSMOTIC FRAGILITY

Normally red cells are suspended in plasma which exerts an osmotic pressure equivalent to 0 85 per cent sodium chloride When placed in hypotonic solutions haemoglobin is freed from the cells but the process is not a simultaneous one some of the cells lysing at one concentration of saline and others at a different strength Arterial blood is less fragile than venous blood which if saturated with CO_2 is markedly fragile This effect is due to alteration in *pH*

Red-cell osmotic fragility is really a measure of spherocytosis since the quantitative fragility is directly proportional to the number of spherocytes but as the test depends on *pH*, oxygen tension temperature the nature of the hypotonic solutions and the proportion of blood used great care must be taken to standardize the technique Normal oxygenated cells begin to lyse at 0 45 per cent sodium *chloride and lysis is complete at 0 3 per cent Spherocytes begin to* lyse at 0 72 per cent and lysis is complete at 0 4 per cent sodium chloride Cells from thalassaemia, sickle cell anaemia obstructive jaundice and some megaloblastic anaemias are resistant to changes in osmotic pressure

The test may be reported as that percentage of saline in which complete lysis occurs or when lysis starts It is best however to report the results graphically (Fig 30) plotting haemolysis percentage against saline percentage From the curve normally sigmoid the median corpuscular fragility (M C F) may be obtained as the concentration of saline causing 50 per cent haemolysis In mild spherocytosis the curve may show a tail indicating a small number of fragile cells Such tails may occasionally be seen in blood from normal infants and it is recommended that a normal blood from a child be tested at the same time

INCUBATION FRAGILITY

The osmotic fragility performed on incubated blood is considerably altered and minor defects are made more obvious Normal

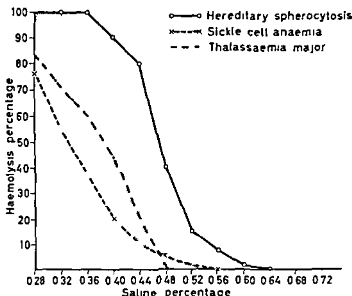


FIG 30 —Osmotic fragility curves the shaded area represents the normal limits

controls must be used in the test It may be necessary to set up concentrations of saline above 0 85 per cent since after incubation some cells are lysed at this strength

MECHANICAL FRAGILITY

The ability of red cells to withstand mechanical trauma is not easily capable of accurate measurement For this reason the procedure is not a routine laboratory investigation except in specialized laboratories Literally the cells are agitated with glass beads and haemolysis measured after a standard time The mechanical fragility is abnormal in spherocytosis sickleaemia and in new born children

ABNORMAL HAEMOGLOBINS

The foetal red cells produce a specialized haemoglobin capable of taking up oxygen at a low tension a process necessitated by the anoxaemic conditions of intra uterine life At birth as much as 90 per cent of haemoglobin is of this type but normally it is replaced by adult haemoglobin within a few months The thalassaemia gene suppresses adult (A) haemoglobin so that more foetal (F) haemo globin persists in the phenotype and simultaneous inheritance of another abnormal haemoglobin results in no A haemoglobin at all being found in the phenotype

Haemoglobins A C D E F G H, I, J K, L, M, N, O P, Q and S have been described, the inheritance of a double dose of abnormal haemoglobin genes or one abnormal for example, C plus the thalassaemia gene producing a haemolytic anaemia of greater or lesser severity known as thalassaemia C haemoglobin disease Sickle haemoglobin was originally termed B haemoglobin The inheritance of a gene for S haemoglobin plus another abnormal haemoglobin results in a larger amount of S haemoglobin appearing in the phenotype with consequent severe symptoms, whereas the sickle cell trait is usually symptomless The inheritance of the abnormal haemoglobins is still not clearly understood, there being some doubt that many of the genes are actually allelomorphs of the gene for A haemoglobin They may in fact be variants of adult haemoglobin

The abnormal haemoglobins have a geographical distribution and may serve as ethnological markers Haemoglobin C does not extend south of the equator and S except in some Indian tribes and parts of Greece betrays Negro ancestry Haemoglobin D has been found in Negroes Sikhs Caucasians Algerians and Indians and E has an Oriental distribution in south east Asia Haemoglobin H seems to be peculiarly Chinese and has been reported in association with Q Haemoglobins C J and K have been reported in Algiers J, K and I in Liberia and O in Indonesia

Haemoglobin H is peculiar in that inclusion bodies stained by brilliant cresyl blue are found in the affected cells, and six sided crystalline structures may be demonstrated in cells containing haemoglobin C A peculiarity of sickle haemoglobin is its association with resistance to infection by malignant tertian malaria Normal A haemoglobin consists of four components A_1, A A_3 and A_4 The A component resembles haemoglobin E in its physicochemical properties and is normally present to about 3 per cent In thalassaemic syndromes however, this component may be increased up to 15 per cent

Electrophoresis

Under the influence of an applied electro motive force charged colloidal particles migrate through the medium in which they are dispersed This is termed electrophoresis or less preferably cataphoresis The rate of migration is known as electrophoretic mobility and under standard conditions is constant for a given colloid Electrophoretic mobility depends on the following three factors

(*a*) The charge on the particle as determined by the *p*H of the

dispersed medium (*b*) the applied E M F and (*c*) the ionic strength of the dispersed medium

Proteins being lyophilic colloid have electrophoretic mobility electrophoresis provides a means of separating the components of such a mixture A mixture of haemoglobins may be separated by paper electrophoresis (Fig 31) in which the pigment mixture is applied across the width of a narrow strip of filter paper saturated with buffer The ends of the strip are placed in troughs of buffer in a Perspex tank and a current allowed to flow through the strip for several hours For comparison normal A haemoglobin or a

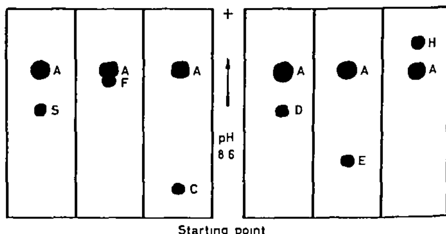


FIG 31 —Diagram showing relative electrophoretic mobilities of abnormal haemoglobins on filter paper at *p*H 8 6

known abnormal haemoglobin are tested under the same conditions This provides reference points to measure the mobilities of the suspected abnormal haemoglobins The separated haemoglobins may be fixed by heat stained and the comparative amounts of each estimated by a scanning apparatus The technique is not an accurate one but is a good routine method of estimating abnormal haemoglobins with an error of only a few per cent

Although all haemoglobin compounds may be used for electrophoresis the *carboxyhaemoglobin pigment appears to be the most* stable and preparations are saturated with carbon monoxide before a run There is however no necessity to fill the tank with the gas

At *p*H 8 6 haemoglobins move toward the anode H and I moving the fastest followed by A but at *p*H 6 5 haemoglobin H moves toward the cathode This property distinguishes H from I Haemoglobins S and D have the same electrophoretic mobility but D does not produce sickling

Alkali denaturation

Foetal haemoglobin differs from other haemoglobins in that it is resistant to alkali denaturation The alkaline haematin produced by the addition of alkali to the haemoglobin solution is estimated by a spectrophotometric method, readings being taken at intervals after the addition of alkali A quicker but less accurate method involves stopping the reaction after one minute with a precipitating reagent the supernatant unchanged haemoglobin being measured as resistant haemoglobin Some loss is to be expected in the first minute A modification of this method uses whole blood instead of a haemolysate

Other methods of identifying abnormal haemoglobins

Paper electrophoresis is a suitable routine technique but fails to differentiate between some haemoglobins Starch electrophoresis provides further information and electrophoresis in agar is also useful especially to separate haemoglobins A and F Ion exchange-resin chromatography is extremely useful, the sequence of mobilities differing markedly from that of paper electrophoresis The solubility of the abnormal haemoglobins at different forms of crystallization is characteristic haemoglobins S and D, for example, being differentiated by this means Haemoglobin H has the property of being denatured in the cold and the precipitated material can then be removed by centrifugalization This provides a means of separating H from a mixture

There seems no doubt that with new and improved techniques more abnormal haemoglobins will be discovered and more particularly, other components of what are at the moment regarded as simple haemoglobins

TECHNIQUE

SERUM BILIRUBIN (VAN DEN BERGH)

The following are details of the Van den Bergh serum bilirubin test

(a) Diazo reagent

(i) Sulphanilic acid 1 0 g
Hydrochloric acid conc 15 ml
Distilled water to 1 000 ml

(ii) Sodium nitrite 20 g in water to make 100 ml Dilute 1 40 before use that is 0 5 ml reagent to 20 ml water

To 10 ml of (i) add 0 3 ml dilute (ii) Use reagent the same day

(*b*) 0 15 N/HCl (15 ml conc HCl diluted to 1 litre with distilled water)
(*c*) Absolute methanol
(*d*) Stock methyl red standard (0 29 g pure methyl red dissolved in glacial acetic acid and volume made up to 100 ml)
(*e*) Working methyl red standard (2 9 mg methyl red per litre at *p*H 4 63) Place 1 0 ml of stock standard in a litre flask together with 5 0 ml of glacial acetic acid Add water to wash 14 4 g of crystalline sodium acetate into the flask. When dissolution is complete make the volume up to 1 litre with distilled water This standard is equivalent to 8 0 mg bilirubin per cent by the method used

Method—Direct bilirubin

Into two small tubes pipette

	Blank	*Test*
Distilled water	4 3 ml	4 3 ml
Serum	0 2 ml	0 2 ml
0 15 N/HCl	0 5 ml	—
Diazo reagent	—	0 5 ml

Mix immediately and allow to stand 15 minutes Read in the photoelectric colorimeter using Ilford spectrum green filter 604

Method—Total bilirubin

Into two small tubes pipette

	Blank	*Test*
Distilled water	1 8 ml	1 8 ml
Serum	0 2 ml	0 2 ml
0 15 N/HCl	0 5 ml	—
Diazo reagent	—	0 5 ml
Methanol	2 5 ml	2 5 ml

Mix immediately and allow to stand 30 minutes Read in photoelectric colorimeter as before

Calculations

$$\text{Direct bilirubin} = \frac{\text{Reading of test} - \text{reading of blank}}{\text{Reading of standard}} \times 8\ 0 \text{ mg per cent}$$

$$\text{Total bilirubin} = \frac{\text{Reading of test} - \text{reading of blank}}{\text{Reading of standard}} \times 8\ 0 \text{ mg per cent}$$

Indirect bilirubin = Total − direct = mg per cent

HAEMOSIDERIN IN URINE

Reagent

Equal parts 2 per cent potassium ferrocyanide in distilled water and N/5 HCl freshly mixed before use

Method

(1) Centrifuge the urine and remove supernatant
(2) Replace supernatant with equal volume of reagent
(3) Resuspend deposit and allow to stand 10 minutes
(4) Centrifuge and transfer deposit to a slide
(5) Place cover glass on deposit and examine

The blue granules of haemosiderin are 1–3μ in size

OSMOTIC FRAGILITY (DACIE)

Reagent

Sodium chloride	180 g
Disodium hydrogen phosphate (anhydrous)	27 31 g
Sodium dihydrogen phosphate (anhydrous)	3 74 g

Dissolve the salts in distilled water and adjust the final volume to 2 litres This is saline buffered to *p*H 7 4 and is osmotically equivalent to 10 per cent sodium chloride The solution keeps well in a stoppered bottle For use dilute with nine times its volume of distilled water making an osmotic 1 per cent solution

Method

(1) Prepare 5 0 ml amounts of saline solutions from 0 72 per cent to 0 28 per cent differing by strengths of 0 04 per cent
(2) Collect blood into heparin and aerate by swirling around in a watch glass until bright red
(3) Using a fresh pipette for each tube wash 50 c mm of blood into each saline dilution
(4) Wash 50 c mm blood into a blank of 5 0 ml 1 per cent saline and the same amount of blood into 5 0 ml distilled water as a standard 100 per cent haemolysis
(5) Mix the contents of all tubes and allow to stand at room temperature for 30 minutes
(6) Centrifuge at 3 000 r p m for 5 minutes carefully remove the supernatant without disturbing the deposit and read in the photo electric colorimeter using an Ilford bright spectrum yellow green filter No 625
(7) Subtract the blank reading (usually nil) from all readings and cal culate haemolysis percentages from the formula

$$\frac{\text{Reading of test}}{\text{Reading of standard}} \times 100 \text{ per cent}$$

(8) Express the results graphically plotting saline percentages on the abscissa and haemolysis percentages on the ordinate

INCUBATION FRAGILITY (DACIE)

Method

(1) Carefully defibrinate blood under sterile conditions
(2) Incubate duplicate 2 0 ml amounts in sterile vials
(3) After 24 hours at 37 C pool the contents of the two vials
(4) Aerate and set up osmotic fragility including saline concentrations of 0 76 0 8 and 0 84 per cent Set up blank in 1 2 per cent saline

TABLE VI

NORMAL RANGE OF INCUBATION OSMOTIC FRAGILITY

Per cent NaCl	*Per cent haemolysis*
0 28	90–100
0 32	80–100
0 36	70–100
0 40	65–100
0 44	56–96
0 48	30–80
0 52	24–72
0 56	5–70
0 60	0–40
0 64	0–19
0 68	0–10
0 72	0–5
0 76	0–2
0 80	Nil

MECHANICAL FRAGILITY

Method

(1) Adjust the packed cell volume of heparinized blood to 45 per cent if necessary using the patient s own plasma
(2) Place 2 0 ml volumes of the blood in 80 × 10 mm tubes and add four glass beads of 4 mm diameter to each tube
(3) Stopper the tubes with rubber bungs and rotate on the suspension mixer at 33 r p m for 60 minutes at room temperature
(4) Pool the contents of two of the tubes and make 1 100 dilutions of the blood in saline and ammoniated water (N/150 ammonia) respectively
(5) Centrifuge the saline tube and read both tubes in the photo electric colorimeter using usual haemoglobin filter

The ammonia represents 100 per cent haemolysis as a standard against which the saline tube is estimated Normal by this technique 2–5 per cent haemolysis

AUTOHAEMOLYSIS (DACIE)

Method

(1) Collect venous blood and deliver without frothing in 1 0 ml amounts into several 80 × 10 mm tubes Allow to clot undisturbed at room temperature

(2) When the clots have started to retract, place one tube in the refrige rator at 4 C and the others in a water bath at 37° C Leave 2 hours and examine

If autohaemolysis is taking place haemoglobin diffuses into the serum at 37 C but not in the control tube at 4° C

RED CELL SICKLING

Method

(1) Mix a drop of capillary blood the size of a pin head with a similar sized drop of freshly prepared 2 per cent sodium metabisulphite

(2) Seal the mixture on a slide under a coverslip and incubate at 37 C

(3) Set up normal blood as a control and examine both after 15 minutes 30 minutes 1 hour and 2 hours

Sickling occurs very rapidly in the homozygote but is slower in the heterozygote with not so many bizarre forms

ACID SERUM TEST (HAM)

Method

(1) Collect venous blood and defibrinate with glass beads Treat normal controls similarly

(2) Separate the cells and wash three times with saline finally making a 5 per cent suspension in saline

(3) Place 1 0 ml of test cells in each of four tubes and similarly treat control cells

(4) Acidify the serum[1] of patient and control by adding 0 05 ml N/5HCl to 0 45 ml serum

(5) To the control cells add 1 0 ml of unacidified test serum

(6) To the first tube of test cells add 1 0 ml of unacidified test serum
To the second tube of test cells add 1 0 ml of unacidified control serum
To the third tube of test cells add 1 0 ml of acidified test serum
To the fourth tube of test cells add 1 0 ml of acidified control serum

(7) Mix the contents of all tubes and allow to stand 1 hour in a water bath at 37 C

(8) Mix again centrifuge and examine for haemolysis In paroxysmal

[1] Serum derived from clotted blood may already show haemolysis in paroxysmal nocturnal haemoglobinuria. It is recommended that the serum be obtained from the defibrinated blood

nocturnal haemoglobinuria none of the control cells with un acidified test serum show evidence of haemolysis Lysis is present in the third tube of test cells but not in any of the others The first tube of test cells may show a trace of haemolysis

N B If marked spherocytosis is present in a positive test repeat the test with serum inactivated at 56 C for 30 minutes

PAPER ELECTROPHORESIS OF HAEMOGLOBINS

The following is the technique for paper electrophoresis of haemoglobins

(*A*) *Preparation of haemolysate*

(1) Collect venous blood into oxalate
(2) Wash the cells once with 0 85 per cent saline
(3) Shake the packed cells for 5 minutes with 1 2–1 8 volumes of dis tilled water (depending on the degree of anaemia) and 0 4 volume of toluene
(4) Centrifuge at 3 000 r p m for 20 minutes and discard the two upper layers of supernatant
(5) Filter the clear red solution and store at 4 C until ready for use

(*B*) *Electrophoresis*

Reagent (*i*) *Barbitone buffer pH* 8 6 *of ionic strength* (I) = 0 05

Barbitone sodium	5 0 g
Hydrated sodium acetate	3 33 g
0 1/N sulphuric acid	34 2 ml
Hydrated copper sulphate	1 0 mg
Distilled water to 1 000 ml	

Reagent (*ii*) *Naphthalene black solution*

Saturated solution of naphthalene black in methanol containing 10 per cent glacial acetic acid

Reagent (*iii*) *Differentiator*

Methanol	660 ml
Glacial acetic acid	60 ml
Normal hydrochloric acid	10 ml
Distilled water	330 ml

Reagent (*iv*) *Clearing agent*

Equal parts of liquid paraffin and 1 bromonaphthalene

Method

(1) Dilute haemolysate with buffer to a concentration of 10 0 g per cent

(2) Saturate the haemolysate with carbon monoxide [1]
(3) Fill troughs of tank with buffer and equalize levels
(4) Draw pencil lines across the filter paper strips (9 cm × 24 cm Whatman No 1 or 3 MM) halfway between the centre of the paper and the cathode end, and mark with identification number
(5) Dip the papers in buffer and blot between blotting paper sheets
(6) Rest papers across the supporting framework with their ends dipping in the troughs ensuring that the starting point is at the cathode end
(7) Allow a current of 240 volts at 4 milliamps per strip to flow for 1 hour
(8) Turn off current and apply haemolysates with a camel hair brush or applicator along pencilled lines to within a few millimetres of each edge of the strips
(9) Re apply potential for 16–20 hours
(10) Remove the strips and dry in air at room temperature
(11) Fix in hot air oven at 110° C for 10 minutes
(12) Stain 8 minutes in naphthalene black solution
(13) Rinse free of excess dye in stain solvent (methanol acetic acid)
(14) Differentiate in many changes of differentiator until background is white
(15) Rinse in water and dry at room temperature
(16) Place in clearing agent until strips are translucent
(17) Scan and estimate areas enclosed by curves with a planimeter

ALKALI DENATURATION

Spectrophotometric method (Jonxis and Visser)

(1) Prepare haemolysate of concentration 10 g per cent and wash 0 1 ml into 10 ml of water to which 2 drops of 10 per cent ammonia have been added
(2) Measure the extinction of this solution in a spectrophotometer at 576 mμ (E_B)
(3) Wash 0 1 ml of the same solution into 10 ml of 0 06 N NaOH and 2 drops of 10 per cent ammonia
(4) Measure the extinction (E_T) of this solution every minute for 15 minutes
(5) Place the solution in a water bath at 37 C for 15 minutes
(6) Cool to room temperature and measure the extinction (E_E)
(7) The percentage of resistant haemoglobin at a certain time is calculated from

$$\frac{E_T - E_E}{E_B - E_E} \times 100$$

(8) Plot the logarithms of the percentages of resistant haemoglobin against time By extrapolation to zero time the percentage of alkali resistant haemoglobin in the original sample can be calculated

[1] Add small amounts of 80 per cent formic acid solution to concentrated sulphuric acid at 60 C This must be done in the fume chamber

ONE MINUTE DENATURATION METHOD (SINGER)

Reagent

(i) 1/12 N NaOH kept in refrigerator in paraffin lined bottle
(ii) Precipitating solution
Half saturated ammonium sulphate solution 800 ml
10 N hydrochloric acid 2 ml
(iii) Toluene haemolysate adjusted to approximately 10 g per cent

(1) Determine exact haemoglobin of diluted toluene haemolysate in photo-electric colorimeter using spectrum yellow green filter 625 and dilution of 1 50
(2) Place 1 6 ml of the alkaline reagent in a small test tube at 20 C
(3) Add 0 1 ml of haemolysate rinsing pipette six times and gently shake the tube for 10 seconds Start stop-watch as haemoglobin is added
(4) At exactly one minute add 3 4 ml of the precipitating reagent invert the tube six times and filter through a double layer of filter paper
(5) Determine haemoglobin value of filtrate and express as a percentage of the initial amount of haemoglobin

N B The reaction on which this test is based is influenced by *p*H temperature and time At *p*H 12 7 and 19 C–21 C all non resistant haemoglobins are denatured to the brownish pigment alkaline globin haematin within one minute The one minute denaturation value of Singer represents the percentage of foetal haemoglobin in the sample but a small amount is destroyed within the first minute making the accurate estimation of small quantities impossible

SOLUBILITY OF REDUCED HAEMOGLOBINS

Reagent

(i) Phosphate buffer
Potassium dihydrogen phosphate (KH_2PO_4) 16 9 g
Disodium hydrogen phosphate (Na_2HPO_4) 17 7 g
Dissolve the salts in distilled water and make up to 100 ml
(ii) Sodium hydrosulphite reducing agent ($Na_2S_2O_4$)

Method

Solubility in 2 24 M phosphate

(1) In a volumetric flask mix 8 0 ml of phosphate buffer and 100 mg sodium hydrosulphite Over this layer 1 ml of water
(2) Add a volume of haemoglobin solution containing 50 mg haemoglobin taking care to avoid precipitation at this stage
(3) Immerse the flask in a water bath at 25 C
(4) Add water to the 10 0 ml mark and completely mix contents by inverting the flask several times

(5) If a precipitate appears centrifuge the mixture at 17 000 × g for 20 minutes
(6) Determine the haemoglobin value of the supernatant solution

N B At phosphate concentration of 2 24 M specimens containing haemoglobin S, precipitate while haemoglobin D remains in solution Haemoglobin H has a low solubility but is differentiated on its electro phoretic mobility

HAEMOLYTIC ANAEMIAS DUE TO AUTO ANTIBODIES

ANTIGEN ANTIBODY REACTIONS

An antigen[1] is a substance usually protein in nature which when injected into an animal lacking that substance, will stimulate the production of a neutralizing substance with which it will react in some observable way The neutralizing substance produced in response to the stimulus of an antigen and which will react with it is known as an antibody A specific antibody will react only with the antigen which called it into being but the injection of a pure antigen may on occasions give rise to antibodies which react with allied antigens Such antibodies are termed non specific or group anti bodies

Antibodies react in many different ways and are named according to their action on cells containing the antigen Agglutinins cause the cells containing the antigen to agglutinate or clump together Lysins are antibodies which liberate haemoglobin from the cells containing the specific substance by causing multiple lesions of the cell envelope Agglutinins in the main exert their effect without the assistance of other serum factors, but lysins always require part of the complement complex for their action However agglutinins and lysins often run together and in fact may be the same antibody reacting in a different manner, or two closely allied forms of the same antibody

Antibodies may be naturally occurring that is they have a place in the normal scheme of things and may be expected in normal indi viduals lacking the antigen, or they may be immune, produced by parenteral administration of the antigen into similar individuals There are physical and chemical differences between the two types of antibody Thus under normal circumstances a naturally occurring antibody reacts best at 4° C and this is termed its thermal optimum Immune antibodies of the same series react best at 37° C , a reversed thermal optimum Not all immune antibodies react best at 37° C

[1] It is suggested that this section be read in conjunction with the appropriate chapters in Section II

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(3) Immerse the flask in a water bath at 25 C
(4) Add water to the 10 0 ml mark and completely mix contents by inverting the flask several times

or albumin These antibodies in consequence are often termed in albumin antibodies Other antibodies may have no visible effect in either saline or albumin but cells in albumin are blocked Such antibodies are termed cryptagglutinoids The effect of blocking antibodies can be demonstrated by Coombs test (*see* below)

Description of antibodies

Chemical terms are used to describe the antibodies a saline or complete antibody being said to be bivalent and an albumin anti body or cryptagglutinoid univalent Antigen antibody reactions are explained diagrammatically in Fig 32

ACQUIRED ABNORMAL HAEMOLYTIC MECHANISMS

Landsteiner s rule states that the antigen and its corresponding antibody cannot exist in the same blood , but under some conditions this law does not hold true The characteristic feature of the acquired haemolytic anaemias is the development of antibodies against the patient s own red cells These auto antibodies are rarely specific but on occasions a specificity for certain of the blood group anti gens may be demonstrated The cause of the abnormal development of auto antibodies is in many cases obscure, such haemolytic anaemias being grouped together as idiopathic The haemolytic process in a second group seems to be initiated by virus infections, particularly virus pneumonia, but infectious mononucleosis, in fluenzae and measles have also been indicated Paroxysmal cold haemoglobinuria is a syndrome forming a third group most cases having a background of untreated or partly treated syphilis but a non syphilitic form with the same type of antibody has been described A fourth group is a heterogenous one associated with advanced cancerous growths leukaemias collagen disorders and diseases of lymphoid tissues Finally a fifth group is formed by a self limiting haemolytic anaemia, the acute cases of which seem to be cured by blood transfusion The collective name for this group is Lederer s anaemia but it is not certain that all cases should be in- cluded since antibody production has not been shown to occur consistently

Blood picture

The acquired haemolytic anaemias have no inherited red cell defect and in fact blood from such a case can be transfused to a

since there are immune substances reacting best at other temperatures, but as a rule such antibodies do not occur naturally

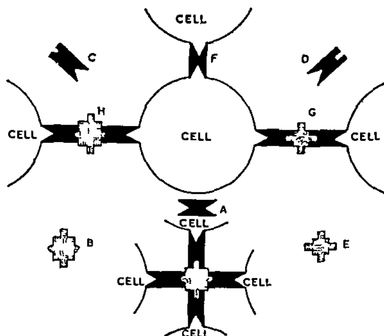


Fig 32 —Antigen antibody reactions A complete (in saline) antibody B anti human globulin C incomplete (cryptagglutinoid) antibody D incomplete (in albumin) antibody E albumin F in saline agglutination G in albumin agglutination H Coombs test agglutination with cryptagglutinoid sensitization The final drawing shows agglutination by anti human globulin of albumin antibody sensitized cells and cryptagglutinoid sensitized cells

Cold antibodies and blocking antibodies

Those antibodies which react best at 4 C are termed cold antibodies and group antibodies among the cold agglutinins are common Immune agglutinins may react in saline, or may not exert their effect at all in this medium In the latter case the cells used may be affected by the antibody in such a way that a good saline acting antibody no longer has any effect on them The cells are then said to be blocked and the antibody is called a blocking or incomplete antibody Such an antibody however may show its agglutinating power if the cells are suspended in a high protein medium such as

the extremities Raynaud s phenomenon in which the affected parts become first white and then purple in colour, is often exhibited by patients Actual agglutination of the red cells takes place in the chilled region

The antibody associated with virus pneumonia is a cold agglutinin sometimes of very high titre Haemolysis, however, does not always occur seeming to be a function of high thermal amplitude rather than titre

THE NATURE OF AUTO ANTIBODIES

Chemically these antibodies are globulins, warm antibodies behaving as γ globulins and cold antibodies mainly as β and α globulins Sera containing cold antibodies do not sensitize cells if heated first but warm antibodies will still affect red cells even after inactivation of the sera at 56° C Unfortunately these criteria do not always hold good some cold antibodies for instance behaving as γ globulins It is useful to examine the reactions of cold anti bodies at different *p*H levels although this procedure may produce results which vary from serum to serum even from patients suffering from the same type of anaemia

Coombs' reagent

Since globulins are protein in nature an antibody can be produced against them and Coombs reagent or anti human globulin serum is a very useful tool in this work Human globulins are injected into the rabbit or goat to produce anti human globulin serum which is absorbed to remove naturally occurring red cell antibodies The anti serum is then standardized by testing against cells which have been sensitized without agglutination The anti human globulin acts as an agglutinin clumping red cells which may have adsorbed antibody onto their surface Anti globulin sera are not specific for any type of globulin reacting with *alpha, beta* and *gamma* globulins A specific antiserum cannot be prepared by using only a specific globulin as an antigen

Anti human globulin serum is easily inactivated by traces of serum or dirty equipment It is essential that the cells under investigation be washed so that the washings tested with salicyl sulphonic acid show no turbidity The reagent may be used in three ways First, to test cells for the presence of adsorbed antibodies This is known as the direct Coombs test Secondly, a suspected serum is allowed to react with appropriate cells which are then tested This is the indirect Coombs test Thirdly, the reagent may be treated with purified

normal recipient when it will be found that apart from an initial small loss the red cells survive normally Spherocytes target cells and schistocytes may, however be seen in the stained blood film which may also show actual clumping of the red cells This is particularly noticeable if cold slides are used to make the blood films The osmotic fragility may be increased but this depends on the number of spherocytes present and this also applies to the incubation fragility The patient is usually jaundiced but in severe cases quite a large amount of direct serum bilirubin may be present because of liver damage Anaemia is invariable and the blood picture one of regeneration An unusual appearance sometimes seen in stained films is erythrophagocytosis the red cells being ingested usually by monocytes The phenomenon is made more obvious by making films from the buffy coat of defibrinated blood thus concentrating the leucocytes

Antibodies in haemolytic anaemia

The auto antibodies of haemolytic anaemia may have a thermal optimum of 4 C 20 C or 37 C They may have a high thermal amplitude that is they react well at different temperatures but are broadly divided into cold and warm antibodies The majority of idiopathic acquired haemolytic anaemias exhibit a warm antibody reacting best at 37 C In this group some specificity has been demonstrated particularly with the Rh blood group system (*see* Section II) A smaller number of idiopathic acquired haemolytic anaemias exhibit cold auto antibodies

Warm antibodies —These circulate in the blood and sensitize the red cells the antibody being of the incomplete or albumin type Most of the sensitized cells lose the antibody and survive normally but those which become loaded with antibody are destroyed in the blood vessels

The Donath Landsteiner antibody responsible for paroxysmal haemoglobinuria is adsorbed onto the red cells in the cold together with complement but the cells are not lysed until more complement is adsorbed in the warmer parts of the body Stagnation of the cells in a chilled part of the body is sufficient to precipitate a haemolytic episode

Cold antibodies —These with a small thermal range causing haemolytic anaemia are sometimes albumin antibodies which may need acidification to show their full effect *in vitro* The patient does not have a severe anaemia and attacks of haemoglobinuria are directly related to chilling of some parts of the body particularly

water bath The blood is allowed to clot at 37° C and serum pipetted off at this temperature If cold antibodies are present some blood is delivered into warm saline and the cells washed in warm saline immediately, if a direct anti globulin test is to be done The patient s serum is titrated against normal and trypsinized Group O cells at different temperatures and an indirect anti globulin test performed on the 37° C titration The Wassermann reaction and Donath Landsteiner are also performed on the serum

Should an antibody be detected either free in the serum or attached to the cells an attempt should be made to classify it and if possible to determine its specificity The anti globulin test at different dilutions and γ globulin neutralization will assist in classifying the antibody as cold or warm but the specificity is often impossible to demonstrate It may be necessary to remove antibody from sensitized cells by elution in order to obtain a potent material with which to work This process is also of great value in separating mixed antibodies The antibody must be tested at the appropriate temperature against cells of known antigenic components and anti globulin tests performed on the cells exposed to the action of the serum

The haemolytic activity of cold or warm antibodies is determined using fresh normal serum as a source of complement to dilute the serum The tests are carried out at the appropriate temperature and acidification of the serum might occasionally help the reaction Cells of paroxysmal nocturnal haemoglobinuria are very sensitive indicators of haemolytic activity but if not available normal Group O cells and trypsinized Group O cells should be used

The haemolytic anaemia produced as a result of foetal sensitization of the mother (haemolytic disease of the newborn) is dealt with in Section II of this book

OTHER HAEMOLYTIC ANAEMIAS

Many drugs and chemicals including phenylhydrazine hydrochloride sulphonamides organic arsenicals many hypnotics and analgesics can cause a haemolytic anaemia but in most cases there appears to be a personal idiosyncrasy In haemolytic disorders due to benzene compounds Heinz bodies may be demonstrated in the red cells These actually consist of denatured globin called verdoglobin which is an intermediate compound between haemoglobin and bilirubin Any substance capable of producing verdoglobin *in vitro* is capable of producing Heinz bodies *in vivo* The blood from a haemolytic anaemia of this type is often brownish in colour and the isolated Heinz bodies green and refractile Heinz

gamma globulin so that it no longer reacts with the warm antibodies In this way it can be used to differentiate between warm and cold antibodies and the test is termed the anti human globulin (A H G) neutralization test An indication that the antibody is of a particular type may be obtained by testing sensitized cells against dilutions of A H G Cells sensitized with cold antibodies are always agglu tinated *most strongly in relatively strong concentration* of reagent whilst those sensitized with warm antibodies are usually agglu tinated best in highly diluted A H G

Controls —These are essential when using anti human globulin serum and should consist of cells deliberately sensitized by a weak incomplete cold agglutinin and others sensitized with an incomplete warm antibody Unsensitized cells are also included to ensure that anti species agglutinins are not active at low dilutions

ENZYME METHODS

If certain cells are exposed to the action of a broth culture of *Vibro cholerae* they become agglutinable by incomplete antibodies Enzymes such as tryspin papain and ficin also have this effect, it being assumed that parts of the red cell envelope are digested off thus exposing further receptors Such procedures are invaluable in the detection of antibodies but sometimes an antibody is encountered which sensitizes so that a positive anti globulin reaction is obtained but does not react visibly with enzyme treated cells On the other hand enzyme treated cells may themselves be sensitized so that a positive anti globulin reaction is obtained Non specific agglutination may occur on over incubation with trypsin and naturally occurring cold agglutinins can interfere with enzyme methods by causing positive results unless the reagents are kept warm

SEROLOGICAL INVESTIGATION OF HAEMOLYTIC ANAEMIAS

Since sufferers from haemolytic anaemia are often more capable of making antibodies than normal individuals one of the first investigations must be to determine as far as possible the blood group genotype (Section II) to avoid further sensitization by in compatible transfusion and to assist in determination of specificity of the antibody Blood is collected from the patient under con ditions which make it difficult for cold antibodies to be adsorbed onto the cells The syringe is warmed and the blood discharged into a tube set in a thermos flask of water at 37° C acting as a portable

INDIRECT COOMBS TEST (TO DEMONSTRATE ANTIBODY IN SERUM)

(1) Place two volumes of suspected serum in a tube and add one volume of 50 per cent suspension of Group O cells of appropriate antigenic structure to catch the antibody
(2) Incubate at 37 C for 2 hours Additional tests must be set up at 20 C and + 4 C
(3) Examine microscopically for agglutination
(4) Wash the cells and perform direct Coombs test

Control with the selected cells and a serum known to react with them and the same cells without serum Agglutination at Stage 3 indicates the presence of saline antibody

GAMMA GLOBULIN NEUTRALIZATION TEST

(1) Make fourfold dilutions of a 4 per cent solution of human γ globulin in saline dilutions from 1 4 to 1 4 096
(2) Dilute a potent anti human globulin serum 1 4 in saline and add equal volume to each of the γ-globulin dilutions
(3) Leave all tubes at room temperature for 10 minutes
(4) Perform direct Coombs tests on cells which have been sensitized by the test serum using the neutralized or partially neutralized anti human globulin serum

Controls

(*a*) Group O Rh positive cells exposed to an incomplete anti D serum (*b*) cells coated with the normal incomplete cold antibody (*c*) Group O unsensitized cells

PREPARATION OF ANTI GLOBULIN SERUM (COOMBS REAGENT)

Alginate method (*Slavin*)

(1) Mix thoroughly 4 ml of 4 per cent sodium alginate solution with 1 ml of sterile human serum of blood Group O
(2) Pour this material into a syringe with a wide bore needle and inject intraperitoneally into a rabbit
(3) With another syringe inject into the same site 2 5 ml of sterile 1 per cent aqueous solution of calcium chloride
(4) Massage the injection site gently but firmly
(5) Three weeks later inject 1 ml of sterile Group O *serum* intra peritoneally
(6) The following day inject 0 2 ml of sterile human Group O serum diluted to 0 5 ml with normal saline into the peripheral ear vein
(7) Nine days after this injection bleed the rabbit for 30–40 ml from the ear vein

body haemolytic anaemia has been described arising without the administration of drugs

Lead poisoning may produce a haemolytic anaemia but the punctate basophilia is usually a good indication that lead is the toxic agent Toadstools Felix mas and other vegetable agents may cause a haemolytic process Baghdad spring anaemia is caused by the susceptible individual inhaling pollen Favism is a similar condition induced by eating the bean Vicia fava

Clostridium welchii infection can cause a haemolytic process but the cause is obvious Blackwater fever is a haemolytic anaemia whose aetiology is bound up with the administration of quinine to patients who are infected with malaria and are therefore already undergoing a haemolytic process Bartonella the cause of Oroya fever can also cause an anaemia of haemolytic type

March haemoglobinuria is caused by a postural defect and is allied to adolescent albuminuria

TECHNIQUE

DIRECT COOMBS TEST (TO DEMONSTRATE ANTIBODY COATED CELLS)

(1) Wash the suspected cells three times with large quantities of normal saline and make a final 50 per cent suspension in saline
(2) Make up dilutions of anti human globulin serum from 1 2 to 1 500 [1]
(3) Scrub an opal tile with soap and a nail brush finally washing with hot water Dry on a towel kept only for that purpose and with a grease pencil mark the tile in squares
(4) In each square place a drop of the appropriate dilution of Coombs reagent and one drop of the cell suspension
(5) Mix with a plastic rod and read after 6 minutes

Controls

(*a*) Group O Rh positive cells exposed to a weak incomplete anti D serum (*b*) Group O cells exposed to an incomplete cold antibody (*c*) Group O cells which are unsensitized

These controls should be washed three times and tested at the dilutions found by experiment to give good results

[1] The weaker dilutions should not be doubling dilution but should be rather closer Dilutions suggested are 1 2 1 4 1 8 1 16 1 3 1 64 1 128 1 00 1 50 1 300 1 350 1 400 1 450 1 500

STANDARDIZATION OF ANTI HUMAN GLOBULIN SERUM

First step with unabsorbed serum

(1) Make doubling dilutions of the serum from 1 2 to 1 512
(2) With each dilution carry out a direct Coombs test against known Group O Rh positive cells sensitized with incomplete anti D serum and Group O Rh positive unsensitized cells

If the titre with the sensitized cells is more than twenty times as great as that with the unsensitized cells proceed to the next step If the titre is too low rest the animals for a few months before giving another course of injections

Absorption of anti human species agglutinins

(1) Wash Group O cells until the washings fail to show turbidity with 25 per cent salicyl sulphonic acid
(2) Add an equal volume of anti human globulin serum to the final packed cells mix and allow to stand at + 4° C for 1 hour
(3) Centrifuge remove the supernatant and repeat the procedure with well washed cells of blood Group A_1B

Determination of optimum titre

(i) *Sensitization of cells by warm incomplete antibody*

(1) Make doubling dilutions of a weak incomplete anti D serum in saline to give 1 0 ml amounts of dilutions from 1 2 to 1 512
(2) Add 0 5 ml of 50 per cent suspension of washed packed Group O Rh positive cells to each tube
(3) Incubate all tubes at 37 C for 30 minutes
(4) Wash the cells of each tube three times with large volumes of saline
(5) Prepare 50 per cent suspensions in saline of the washed cells

(ii) *Sensitization of cells by incomplete cold antibody*

(1) Obtain six different samples of blood which have been allowed to stand in the refrigerator overnight and discard the serum in the cold
(2) Wash the loose cells of the clots three times in cold normal saline and make up to 50 per cent suspensions in saline
(3) Test the cells with a known potent anti human globulin serum by direct Coombs method

Usually one or more specimens will prove to have adsorbed incomplete cold antibody

(iii) *Titration of anti human globulin serum*

(1) Scrub clean a large opal tile and mark in squares with a grease pencil
(2) Prepare doubling dilutions of the anti human globulin serum from 1 2 to 1 512 so that 1 0 ml of each dilution is available

(8) When the blood has clotted separate with the aid of a plastic rod and allow to stand overnight at room temperature
(9) Separate the serum and inactivate complement at 56 C for 30 minutes

Alum precipitate method (*Proom*)

Reagent (1) Stock merthiolate solution

Merthiolate (Lilly)	0 25 g
Borax	0 35 g
Sterile distilled water to 25 ml	

This keeps well in the dark for one month

Reagent (2) Merthiolate saline

Stock merthiolate solution	1 part
Sterile normal saline	99 parts

Reagent (3) Alum solution

Potassium alum (KAl (SO_4) 12 H_2O)	25 g
Sterile distilled water to	250 ml

Reagent (4) 5N sodium hydroxide

Sodium hydroxide (NaOH)	10 g
Sterile distilled water to	50 ml

Reagent (5) B D H Universal Indicator

Method

(1) Dilute 25 ml of sterile Group O serum with 80 ml distilled water
(2) Add 90 ml of 10 per cent potassium alum solution
(3) Adjust reaction to *p*H 6 5 with 5N sodium hydroxide using B D H Universal Indicator Add alkali drop by drop since the *p*H changes suddenly The test is done on a tile
(4) Centrifuge the mixture and remove the supernatant
(5) Wash the precipitate twice with 200 ml of 1 10 000 merthiolate saline
(6) Make up the final washed precipitate to 100 ml with fresh merthiolate saline The material may be stored at 4 C for at least 14 days
(7) Inject 5 ml of antigenic material intramuscularly into each buttock of selected rabbits and repeat 14 days later
(8) Bleed for 30–40 ml from the ear vein 9–10 days later
(9) Separate serum overnight at room temperature and inactivate at 56 C for 30 minutes

N B Since not all rabbits produce good antibodies six animals should be injected at the same time when producing anti human globulin serum by any method Anaphylactic shock may kill the animals and adrenalin should be at hand to counteract such ill effects of the injections

(3) After 30 minutes place all tubes in the water bath at 37 C and resuspend the cells
(4) After one hour inspect for haemolysis

COLLECTION OF DEFIBRINATED BLOOD

(1) Collect blood into a small flask equipped with a wooden applicator to which is attached several paper clips bundled together
(2) Rotate the flask so that the blood is swirled against the paper clips until the fibrin clot is seen firmly attached
(3) Remove the clot carefully leaving the free cells and plasma

This technique may be used for many investigations including examination for lupus erythematosus cells (Chapter 11) and preparation of cells for elution

ELUTION OF ANTIBODIES (WEINER)

(1) Collect defibrinated blood and measure volume of cells remaining after removal of clot
(2) Wash the cells three times with normal saline pack and remove supernatant
(3) Cork the tube and place in refrigerator at − 20 C until cells are lysed
(4) Thaw the preparation and when just thawed add ten times the original volume of 50 per cent (v/v) ethanol pre cooled to at least − 6 C
(5) Mix thoroughly by inverting the tube at once several times
(6) Return to refrigerator for 2 hours
(7) Centrifuge at 3 000 r p m for 10 minutes remove supernatant as completely as possible and replace with distilled water
(8) Break up sediment with a pointed plastic rod and mix thoroughly
(9) Centrifuge again remove distilled water and replace with normal saline
(10) Mix sediment with saline and incubate in water bath at 37 C for 30–60 minutes
(11) Centrifuge and remove supernatant which is kept frozen solid until ready for use The eluate is often brown with haemoglobin but should be clear

Normal serum may be used instead of saline for the final solution of the antibody The eluates keep well frozen solid and may be used instead of serum in any of the usual techniques

(3) Place one drop of each dilution in each of the twelve vertical squares on the tile
(4) In the top row of squares place one drop of red cell suspension sensitized with anti D diluted 1 2 Repeat the procedure in the next row with dilution 1 4 then 1 8 1 16 1 32 1 64 and 1 128
(5) In the eighth row place one drop of cells sensitized with cold anti body and repeat in the ninth row with cells sensitized with another cold antibody
(6) In the tenth eleventh and twelfth rows place the appropriate drops of unsensitized A B and O cells
(7) Mix each suspension with a plastic rod rinsing with saline between each square Allow to stand one minute
(8) Rock the tile gently and allow to stand a further minute
(9) Rock and read after 4 minutes

AGGLUTININ TITRATION

The method of determining agglutinin titration is as follows

(1) Make doubling dilutions of the suspect serum in saline
(2) Add an equal volume of 2 per cent cell suspension and incubate 2 hours
(3) Read macroscopically and microscopically
(4) Perform direct Coombs tests on cells if incomplete antibody is suspected

The tests should be performed at 4 C 20 C 30 C and 37 C

The patient s own cells normal Group O cells and trypsinized normal Group O cells should all be tested against the serum

DONATH LANDSTEINER PRESUMPTIVE TEST

(1) Warm two tubes to 37 C and deliver the same amount of blood into each
(2) Place one tube in the water bath at 37 C and the other in crushed ice for 30 minutes
(3) Replace the cold tube in the 37 C water bath without disturbing the clot
(4) When the clots have retracted examine the serum for haemolysis

In paroxysmal cold haemoglobinuria the chilled sample shows haemo lysis but the other sample is clear

Titration of Donath Landsteiner antibody

(1) Make doubling dilutions of the suspect serum in fresh normal serum
(2) Add an equal volume of 4 per cent suspension of washed Group O cells to each tube and immerse the tubes in crushed ice

Reagent (2) Buffer *p*H 7 3

0 947 per cent disodium hydrogen phosphate (Na_2HPO_4)	3 parts
0 907 per cent potassium dihydrogen phosphate (KH_2PO_4)	1 part

Reagent (3) Buffered saline

Buffer *p*H 7 3	1 part
Normal saline	9 parts

Method —Details of the papain method are as follow

(1) Dilute papain with nine volumes of buffered saline
(2) Add one volume of washed packed cells to two volumes of buffered saline papain
(3) Incubate in water bath at 37° C for 30 minutes
(4) Wash the cells twice in saline and make up to strength of 5 per cent in saline

The cells are used as for the trypsin method

An alternative method in which papain is combined with an activator is given in Chapter 17 Pre treatment of cells is unnecessary in this technique

Ficin method

Reagent (1) Ficin suspension[1]

Ficin (Merck)	25 mg
Iso osmotic phosphate buffer *p*H 7 3–7 5	2 5 ml

Reagent (2) Buffer

2 34 per cent sodium dihydrogen phosphate (NaH_2PO_4 $2H_2O$)	20 parts
1 63 per cent disodium hydrogen phosphate (Na_2HPO_4)	80 parts

Method —Details of the ficin method are as follow

(1) Wash the red cells three times in saline and make up to 2 per cent suspension in saline
(2) To nine parts of cell suspension add one part of ficin suspension
(3) Allow to stand 15 minutes at 37° C
(4) Re wash cells twice with saline

The cells must be made up daily subsequent treatment being as for the trypsin method

HAEMOLYSIN TECHNIQUE

(A) (1) Make doubling dilutions of the suspect serum in fresh acidified normal serum (add one tenth part of N/4 hydrochloric acid)

[1] This reagent will destroy the mucous membranes of the mouth if accidentally sucked up by pipette

ENZYME TECHNIQUES

Trypsin method

Reagent (1) —Crystalline trypsin (Armour) 0 1 g
N/20 hydrochloric acid 10 ml

or

B D H trypsin powder 2 5 g
N/20 hydrochloric acid 10 ml

Shake at intervals and after 24 hours at 4 C centrifuge and use supernatant as reagent

Reagent (2) —M/10 phosphate buffer *p*H 7 7
1 63 per cent disodium hydrogen phosphate ($Na\ HPO_4$) 90 5 parts
2 34 per cent sodium dihydrogen phosphate ($NaH\ PO_4\ 2H_2O$) 9 5 parts

Method —Details of trypsin method are as follow

(1) Dilute trypsin with nine volumes of buffer
(2) Add one volume of well washed packed cells[1] to four volumes of diluted enzyme
(3) Incubate in water bath at 37° C for 30 minutes
(4) Wash the cells twice in saline and make up to a strength of 5 per cent in saline These treated cells may be kept up to 48 hours
(5) Make dilutions of suspected serum from 1 in 1 to 1 in 256 and warm to 37 C
(6) Warm cell suspension to 37 C and add an equal volume to each serum dilution
(7) Incubate one hour at 37 C and examine macroscopically and microscopically for agglutination

N B Agglutination is usually quite obvious macroscopically but non specific agglutination may occur if incubation is prolonged beyond the stated time It is essential that the reagents are kept warm since cold agglutinins may give false results

Papain method

Reagent (1) Papain suspension
Papain 1 0 g
Normal saline 100 ml
Shake frequently and allow to stand overnight at 4 C
Decant the supernatant and keep frozen solid at − 20 C
The reagent may be kept one month

For screening purposes cells of Group OR R (CDe/cDE) re used (*see* Ch pter 17)

CHAPTER 9

THE HAEMORRHAGIC AND PURPURIC DISORDERS

INTRODUCTION

THE CIRCULATORY SYSTEM in man consists of the heart and a series of arteries capillaries and veins the intimal coats of which are non water wettable Inherited or acquired abnormalities of the vessels or the blood itself can give rise to uncontrolled haemorrhage from mucous membranes or into the skin and solid organs with slight or no obvious trauma Bleeding disorders are due to a failure of one or more (commonly a combination) of the mechanisms which normally control haemorrhage

SHOCK

The actions of the two sides of the heart are so synchronized that the sudden lowering of input which occurs in acute haemorrhage can cause severe cardiac failure To maintain an adequate input the body reacts to haemorrhage by contracting surface and smaller vessels so that only the larger vessels are blood filled This has the additional effect of cutting down the blood supply to the bleeding point The patient suffers from oligaemic shock varying in severity from a faint to profound collapse The condition may be transient but usually lasts long enough for other mechanisms to function in the control of haemorrhage Profound shock may pass into coma and death, the amount of blood loss not always being the prime factor

The vasoconstrictor effect of shock is produced locally when capillaries are damaged by a needle puncture the cut vessels con tracting for a period of 15–30 seconds thus preventing blood reach ing the injured part The ability of normal capillaries to react to trauma in this way has an effect on the time taken for bleeding to cease from a needle puncture This is known as the bleeding time and depends on many other factors

CAPILLARY ABNORMALITIES

Inability of the capillaries to contract may result in severe haemorrhage from trivial injuries Von Willebrand s disease (*syn*

(2) Add one volume of 2 per cent suspension of normal Group O cells to each tube

(3) Repeat the titrations and incubate one set at 37 C and the other at 15 C for 2 hours

(B) (1) Make doubling dilutions of the suspect serum in fresh normal unacidified serum

(2) Add one volume of 2 per cent suspension of Group O cells from a patient suffering from paroxysmal nocturnal haemoglobinuria [1]

(3) Incubate 2 hours at 20 C

DEMONSTRATION OF HEINZ BODIES

(1) Mix equal volumes of 0 5 per cent methyl violet in saline and blood obtained from a finger prick

(2) Allow mixture to stand in a small tube 10 minutes at room temperature

(3) Make films from the mixture

(4) Expose to formalin vapour 5-10 minutes

(5) Wash thoroughly in distilled water

(6) Counterstain one minute in 0 1 per cent safranin Heinz bodies are stained intense purple

[1] P N H cells a e very sens t e to haemolysis

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CAPILLARY ABNORMALITIES

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constitutional thrombopathy, vascular pseudohaemophilia) is an inherited condition which in part is due to such a dysfunction Transmitted as a sex linked dominant characteristic it produces bleeding from nose gums vagina and other mucous membranes but haemorrhage into joints is uncommon The capillaries are often bizarre and distorted but factors other than vascular abnormalities are also involved

Hereditary haemorrhagic telangiectasia is another inherited anomaly of the capillaries transmitted as a simple Mendelian dominant The telangiectases consist of groups of dilated capillaries the lesions appearing bright red or purple and varying in size from 1 to 3 mm in diameter They are found early in life but increase in numbers with age the haemorrhagic tendency often not appearing until middle age The lesions are found on the face but are more common on mucous membranes The twisted capillary loops may be studied by rendering the skin semi transparent with cedarwood oil and using a microscope with vertical illumination The bleeding time is not increased unless an actual lesion is punctured when it will be found that the cut vessels fail to contract normally

THE PURPURAS

The capillary endothelium can be damaged by toxins viruses bacteria allergy drugs and so on so that it can no longer hold blood under any increased pressure The patient suffers from purpura which manifests itself as numerous haemorrhagic spots in the skin due to effusion of blood from the junction of capillary loops and arterioles This symptom may be present alone or be accompanied by other abnormalities

A deficiency of vitamins C and P such as occurs in scurvy results in faulty synthesis of cement substance of the capillary endothelium Purpura is produced by the increased permeability of the capillary wall

Senile and orthostatic purpura

In old age confluent brownish patches may be found on the back of the wrists and hands *This is termed senile purpura and is due to* increased fragility of the vessel walls The same appearance may be seen on the lower limbs especially in those sufferers from varicose veins and is then sometimes termed orthostatic purpura In infectious diseases especially smallpox the rash may become haemorrhagic because of the damaged capillary endothelium A purpuric condition has also been described in diabetes uraemia hypertension and other conditions

Henoch and Schonlein purpuras

Henoch and Schönlein purpuras are allergic conditions with increased capillary permeability Henoch s purpura occurs in children and adolescents and is associated with gastro intestinal symptoms while Schonlein s purpura occurs in young adults and is associated with joint effusions Since the clinical distinction between allergic purpuras is slight, the group is commonly known as anaphylactoid or Henoch Schönlein purpuras

CAPILLARY RESISTANCE TEST

To uncover capillary disorders use is made of the capillary resistance test also known as Hess Rumpel Leede Weil Grocco-Frugoni or tourniquet test The intracapillary pressure is raised by constriction of the veins with the production of purpuric spots where capillary permeability is increased The lesions produced result from exudation of blood from the arteriolar end of the capillary loop where the arteriole dilates to form the loop (Fig 33) The type of lesion seen microscopically varies with the disorder but it is not possible to make a diagnosis on this test alone

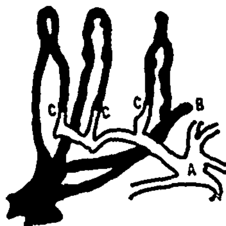


FIG 33 —Skin capillaries (*modified from Humble*) A terminal arteriole B collecting venule C arteriole capillary junction

THE PLATELETS

The platelets are intimately concerned with the control of haemorrhage one of the functions being to reinforce the inherent ability of the capillaries to contract This is done by means of the liberation of serotonin a physiological vasoconstrictor The effect of serotonin may last 30 minutes or longer allowing sufficient time for normal blood to clot Another function is the mechanical one

of plugging small wounds which the platelets are enabled to do by reason of stickiness causing them to adhere together on flowing over a water wettable surface A third property of the platelet is concerned with the production of clotting factors and acceleration of the reaction and yet another function allows healing of a wound or recanalization of a vessel to take place by causing retraction of blood clot This occurs when the platelets enmeshed in the clot send out fine fibrils which attach themselves to strands of fibrin and then contract literally pulling the clot in on itself and allowing clear serum to exude This phenomenon of syneresis or clot retraction is a function of platelet numbers retraction being poor with a soft friable clot if platelets are deficient

Thrombocytopenia may be primary (idiopathic) or secondary differentiating those conditions in which megakaryocytes are present in the marrow from those in which they are absent Primary thrombocytopenia may even show increased production of megakaryocytes but usually there is some defect of maturation evidenced by lack of cytoplasm granularity Thrombocytopenia is often associated with a positive Hess test

Quantitative platelet deficiencies

Idiopathic thrombocytopenia (*syn* Werlhof s disease essential thrombocytopenia) is characterized by petechiae or ecchymoses in the skin as well as haemorrhage from mucous membrane There may be spontaneous remissions and relapses The bleeding time is increased clot retraction poor and Hess test positive The megakaryocytes often show a maturation defect which is due to splenic inhibition the spleen inducing a hormonal effect on the cells Such hypersplenism may occur in a variety of disorders splenectomy producing temporary or permanent cure The marrow may show eosinophilia in addition to faulty megakaryocytes About 50 per cent of cases show anti platelet antibodies

Many drugs produce a primary thrombocytopenia and some cases of this condition can be shown to be associated with antibodies active against platelets It has been suggested that the drug confers antigenic properties on the platelet which in contact with the antibody forming tissues of the spleen gives rise to a specific antibody which attaches itself to the platelet The antibodies can be demonstrated as agglutinins or by the anti human globulin reaction

A rare form of thrombocytopenia is associated with multiple thromboses Thrombotic thrombocytopenia purpura is a disease of unknown aetiology platelets being almost completely absent from the peripheral blood yet the thrombi contain platelets It is

believed to be primarily a vascular condition It is associated with haemolytic anaemia and nervous disturbance

Secondary thrombocytopenias are found as part of many blood disorders The leukaemias pernicious and aplastic anaemias, bone marrow metastases and storage reticuloses can all produce the condition

Qualitative platelet deficiencies

Hereditary haemorrhagic thrombasthenia (Glanzmann) a dis order transmitted as a Mendelian dominant, is characterized by purpura prolonged bleeding time poor clot retraction but no reduction in platelets The platelets may be abnormal large and irregular forms being seen in the peripheral blood The haemorrhagic defect is in the platelets themselves which fail to take part in the clotting mechanism by reason of deficiency of one or more factors

THE BLEEDING TIME

This test depends upon four factors (*a*) Ability of capillaries to contract on injury (*b*) number of platelets (*c*) ability of platelets to clump and adhere closely (*d*) health of the capillary endothelium

Depending on so many factors the test alone does not yield a great deal of information but taken in conjunction with a platelet count and capillary resistance test is of primary value in the investigation of the haemorrhagic disorders It is difficult to standardize a technique for the bleeding time the main lines of standardization being in type and size of needle and depth of puncture Whatever technique is used should be stated in the report and the normals for the method also reported

BLOOD COAGULATION

Coagulation is the process by which fluid blood becomes a firm jelly like material which occludes the bleeding point The clot con sists of a mesh of fibrin in which is caught up most of the red and white cells and platelets Numerous theories of blood coagulation exist and probably the simplest is that of Morowitz

Prothrombin + calcium ions + thromboplastin = thrombin

Thrombin + fibrinogen = fibrin

Prothrombin is a white amorphous glycoprotein normally present in the plasma to a strength of 20 mg *per cent* It is either produced in the liver from vitamin K which *in turn* is synthesized by putrefactive bacteria in the gut or alternatively the vitamin K may be merely stored in the liver the prothrombin being produced in the reticulo-endothelial system outside the liver

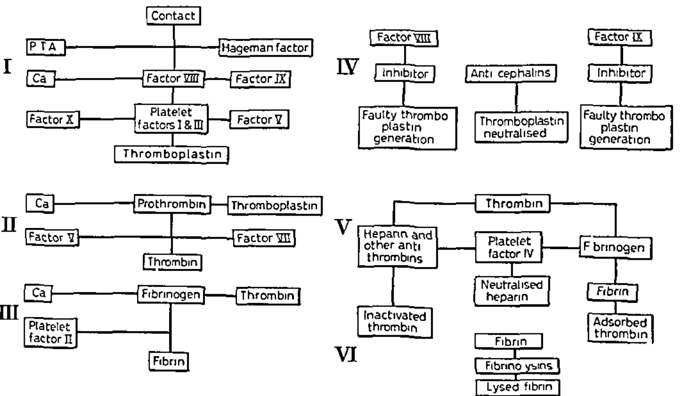


FIG 34 —The interaction of the blood clotting factors

Thromboplastin is the name given to complex substances which can be extracted from many tissues, particularly lung and brain The blood makes its own thromboplastin when it is shed and it is a very potent thromboplastin indeed

Thrombin is a very active enzyme which is capable of converting many hundred times its own weight of fibrinogen to fibrin During the clotting process it is produced quantitatively from prothrombin

Fibrinogen is one of the plasma proteins normally present to a concentration of 0 2–0 5 g per cent in plasma

Calcium ions occur normally in all tissues including the blood

The theory of Howell went one stage further in an attempt to explain the presence of heparin in the blood Heparin is a physiological anticoagulant produced in the liver, lungs and elsewhere and acts by reason of its anti thrombin activity Howell postulated that prothrombin and calcium produced thrombin but when heparin was present no thrombin was formed His view was that thrombo plastin neutralized heparin thus allowing thrombin to be formed

The modern theory or aggregation of theories (Fig 34) assumes that several factors must first react together to produce thrombo plastin, deficiency of one or more of the factors producing an in efficient thromboplastin Clotting still takes place but is prolonged and prothrombin is not fully utilized The clotting time measures the speed of thromboplastin formation and subsequent conversion of prothrombin to thrombin then fibrinogen to fibrin

THE COAGULATION MECHANISM (Table VII)

In the present state of knowledge of the subject it is not possible to be dogmatic regarding the clotting process Blood coagulation occurs in a series of steps the intermediate factors so produced being necessary for subsequent stages but since many coagulation experiments are capable of different interpretations the exact func tion of some factors is at present unknown It must be stressed therefore that the explanatory notes below comprise an attempt to present the process for study rather than a confirmed exposition of the coagulation mechanisms

Intrinsic (blood) thromboplastin generation

(a) On contact with a water wettable surface Factors VIII and IX react with calcium to form an intermediate product of thrombo plastin The exact nature of the contact phenomenon is unknown but Hageman factor and PTA would also appear to be involved

(b) In the presence of calcium ions the intermediate product of the

TABLE VII

SYNONYMS USED FOR FACTORS INVOLVED IN BLOOD COAGULATION

A PREPARATIONS OF THROMBOPLASTIC ACTIVITY

Intrinsic thromboplastin	*Extrinsic thromboplastin*	*Platelet extract*	*Russell viper venom*
Thromboplastin Blood thromboplastin Plasma thromboplastin Complete thromboplastin	Tissue thromboplastin Incomplete thromboplastin	Thrombokinin thrombokinase cytozyme	R V V Thrombokinase Incomplete thromboplastin

B PLASMA AND SERUM FACTORS

Factor V (Owren)	*Factor VII (Koller)*
Prothrombin accelerator (Fonti and Nance) Pro-accelerin → accelerin (Owren) Ac globulin (Ware and Seegers) Thrombogen (Nolf) Prothrombin A (Quick) Labile factor (Quick)	Prothrombin conversion factor (Owren and Bollman) Serum prothrombin conversion accelerator (Alexander and de Vries) S P C A Pro-convertin → convertin (Owren) Co-thromboplastin (Mann) Co-factor V (Owren) Stable factor (Stefanini)
Factor VIII (Koller)	***Factor IX (Koller)***
Anti haemophilic globulin (Lewis and and his colleagues) A H G Anti haemophilic globulin A (van Cramer) Thrombocytolysin (Brinkhaus) Thromboplastinogen (Quick) Plasma thromboplastic factor (Stefanini) P T F Platelet co factor I (Seegers)	Christmas factor (Biggs) Plasma thromboplastin component (Aggeler) P T C Anti haemophilic globulin B (van Cramer) Platelet co factor II (Seegers)
Factor X (Koller)	***P T A (Rosenthal)***
Stuart factor (Hougie and his colleagues) Prower factor (Telfer and his colleagues)	Plasma thromboplastin antecedent (Rosenthal)
Hageman factor	***Prothrombin***
—	Thrombozyme (Nolf) Prothrombin B (Quick)

preceding step activates platelets to liberate several factors four of which are known to be involved in the clotting process

(c) Platelet Factor I and Factor V of the plasma which resemble one another in activity combine with the original intermediate product in the presence of calcium ions the process possibly being initiated by the thromboplastic Platelet Factor III Factor X may also be involved in this step the final product of which is intrinsic thrombo plastin

Prothrombin conversion

(a) In shed blood prothrombin is converted quantitatively into throm bin by the action of intrinsic thromboplastin in the presence of calcium ions Accelerating factors are not required in this reaction

(b) Using extrinsic (tissue) thromboplastins the speed of prothrombin conversion depends upon the plasma concentrations of Factors V VII and X Tissue thromboplastins lack Factors V VII and X and Russell s viper venom lacks Factor X

Fibrinogen conversion

The conversion of fibrinogen to fibrin is an enzymatic function of thrombin with the aid of Platelet Factor II

Thromboplastin inhibitors

(a) Interference with the early stages of the clotting process results in impaired thromboplastin generation Deficiency of Factors VIII or IX can sometimes be shown to be accompanied by inhibitors of the reaction between these factors Such inhibitors are termed circulating anticoagulants

(b) Anti-cephalins have the property of being able to neutralize small amounts of thromboplastin This is probably due to the lipoid part of tissue thromboplastin being related to cephalin

Inhibitors of the prothrombin thrombin reaction

(a) Heparin interferes with the prothrombin thrombin reaction but requires a co factor which is present in plasma albumin The co factor may actually be present together with heparin in basophils The activity of heparin is minimized by the Platelet Factor IV

(b) A natural antithrombin exists in the plasma albumin Its effect is enhanced in any condition in which heparin is increased

(c) Some thrombin is absorbed on to fibrin and can be eluted still in an active form

(d) Hyperproteinaemias can interfere with this stage of the clotting process possibly by adsorption on to thrombin

The fibrinogen fibrin reaction

A normal fibrinolytic process is essential to remove deposition of fibrin from vessel walls but pathological processes can increase the circulating fibrinolysin titre to haemorrhagic levels

THE CLOTTING FACTORS

The factors involved in the clotting process have been described by different workers under various names A list of these names is shown in Table VII It will be noted that there is a tendency to identify the factor by the name of the patient suffering from the deficiency

Factor V is a plasma factor which deteriorates on storage hence is often known as the labile factor (Quick) It is necessary for the production of thromboplastin *in vivo* but then takes no further part in the reaction It is derived from an inactive precursor in the plasma

Factor VI was the original name given to a thromboplastin inter mediate product but since the descriptions were so vague the term is no longer used

Factor VII is an accelerator of the prothrombin conversion using tissue thromboplastin Its function *in vivo* would appear to be connected with bleeding control in tissue since it is not needed in the clotting of shed blood

Factor VIII is a plasma globulin which has been purified and is available commercially It is the factor deficient in haemophilia

Factor IX or *Christmas factor* is deficient in the haemophiloid like disorder Christmas disease and is present in serum

PTA or plasma thromboplastin antecedent is found in serum and plasma and a few families have been described as affected by an haemorrhagic disorder due to its deficiency

Hageman factor was named after two sisters who were affected by an haemorrhagic disease

Factor X is deficient in some patients who exhibit a prolonged clotting time but no evidence of bleeding into tissues It may be identical with Stuart and Prower factors which again were named after affected patients

THE CLOTTING TIME

Since the clotting time measures the speed of several reactions it is not diagnostic of a specific deficiency Where thromboplastin com ponents are deficient the time is prolonged but very few platelets are

required to initiate the clotting process, hence in all but complete thrombocytopenias the clotting time is within normal limits The time is unaffected by deficiencies of the prothrombin–Factor V and VII complex but prolonged with insufficient fibrinogen Slight deficiencies of the essential factors may not greatly affect the test but can be made more obvious by using siliconed glassware By this means the normal clotting time is prolonged and times of abnormal bloods greatly prolonged To obtain consistent results standardization of clotting time techniques is essential

FIBRINOPENIA

A deficiency of fibrinogen may be inherited or acquired Inherited possibly as a simple Mendelian recessive the disorder is rare and unaccompanied by other deficiencies Blood so affected will not clot or if it does the clot is thin and wispy

A constitutional fibrinopenia has been described in women but only in a few cases and I have seen a woman with an apparent cyclical fibrinopenia associated with the onset of menstruation

Acquired fibrinopenia is usually only partial and is associated with severe liver disease carcinoma and infections The fibrinopenia may be due to inadequate production excessive utilisation or destruction by fibrinolysis (*see* below)

An acute defibrination syndrome associated with surgical procedures and more commonly obstetrical abnormalities has been described by many authors It would seem that during surgical *operations and where the placenta is necrosed actual tissue fragments* enter the circulation where they act as thromboplastins The slow coagulation which occurs results in fibrin being deposited upon the cell walls with a compensatory increase in fibrinolysins Thus fibrinogen is both used excessively and destroyed resulting in an haemorrhagic tendency which is often fatal

FIBRINOLYSIS

Fibrin itself can be lysed by fibrinolysins normally present in the circulation where they fulfil the important function of removing fibrin deposited upon vessel walls Fibrinolysis can be increased in anxiety states by severe exercise or the injection of adrenalin The active fibrinolysin is termed plasmin and is derived from a globulin precursor termed plasminogen This conversion is inhibited by an anti plasmin in the albumin fraction of the serum It would appear that some materials are capable of removing the inhibitory effect of anti plasmin thus allowing the formation of active plasmin *in vivo*

Various bacterial filtrates (streptokinase staphylokinase) are

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CLOT RETRACTION

(1) Withdraw venous blood into a dry syringe and place in the special tube in the water bath at 37 C
(2) Note the time taken for the blood to clot and exactly one hour afterwards carefully free the clot and remove on the glass rod
(3) Drain the clot into the tube and measure the volume of serum

The lower limit of normality is 44 per cent of serum The tube is a centrifuge tube with a glass rod set in the cork The rod is roughened with projections to hold the clot so that it can be lifted from the tube

FIBRINOLYSIS

Reagent (*a*) Veronal buffer *p*H 7 35

0 1/M sodium diethyl barbiturate	570 ml
0 1/M hydrochloric acid	430 ml
Sodium chloride	5 67 g

Reagent (*b*) 0 9 per cent sodium chloride in distilled water

Reagent (*c*) Thrombin topical (Parke Davis) 20 units/ml in saline

Reagent (*d*) Buffered saline prepared by mixing equal parts (*a*) and (*b*)

Method

(1) Collect venous blood and mix 9 parts with 1 part 3 8 per cent sodium citrate
(2) Prepare a dilution of 1 16 of the citrated plasma by adding 0 4 ml plasma to 6 ml of buffered saline
(3) Prepare the following tubes in duplicate
   (i) 1 6 ml plasma 1 16
   (ii) 0 8 ml plasma 1 16 + 0 8 ml buffered saline
   (iii) 0 4 ml plasma 1 16 + 1 2 ml buffered saline
   and to each tube of one set add one drop of thrombin and to each tube of the second set 0 1 ml M/20 $CaCl_2$ Mix the tube by inverting once
(4) Place all six tubes in water bath at 37° C together with control tubes prepared from normal plasma
(5) Note the clotting times and leave for 24 hours observing degree of lysis if any at intervals

N B The fibrinolytic enzyme is not stable on storage and the test should be performed at a standard time after collection of blood for this reason

PLATELET ANTIBODIES (KISSMEYER AND NIELSEN)

Reagent (*a*) Platelet extract

(1) Collect 9 parts of blood into 1 part of 10 per cent disodium citrate from a Group O donor
(2) Centrifuge 15 minutes at 1 500 r p m and take off the supernatant

(3) Centrifuge plasma 30 minutes at 3 000 r p m and resuspend platelets in saline to give concentration of 600 000 per c mm
(4) Freeze and thaw three times and finally centrifuge 30 minutes at 3 000 r p m
The supernatant constitutes platelet extract

Reagent (b) *Buffered platelet extract*
To platelet extract add equal volume of buffered saline *p*H 6 5

Reagent (c) Tanned cells
(1) Collect defibrinated blood from a Group O donor and wash the cells three times in neutral saline
(2) Make up 2 per cent *suspension in buffered saline p*H 7 2 *and* add an equal volume of freshly prepared 1 40 000 tannic acid
(3) Incubate at room temperature 10 minutes wash once in buffered saline and make up to 2 per cent in normal saline

Reagent (d) Coated tanned cells
(1) To the tanned-cell suspension add an equal volume of buffered platelet extract
(2) Incubate 10 minutes at room temperature
(3) Centrifuge 5 minutes at 1 000 r p m and wash twice in normal saline containing 1–2 per cent normal human serum to protect against haemolysis
(4) Make up to 1 per cent suspension in normal saline

Reagent (e) Buffered saline *p*H 7 2
Mix 4 volumes 0 8 per cent saline and 1 volume of M/15 Sorensen Buffer *p*H 7 2

Reagent (f) Buffered saline *p*H 6 5
Mix equal volumes saline and Sorensen Buffer *p*H 6 5

Method

(1) Make doubling dilution of patient s inactivated serum
(2) *Add one volume of tanned coated cells* shake and leave at room temperature 30 minutes
(3) Shake again and leave 2 hours at room temperature
(4) Read on agglutinate pattern and shake again
(5) Read again after 18 hours at $-4°$ C

Control with non-coated tanned cells and normal sera with coated tanned cells

THE PROTHROMBIN COMPLEX

Although some workers have shown that purified prothrombin may be converted *in vitro* without the assistance of other blood factors tests of clotting *efficiency demonstrate an intimate* relationship

between prothrombin and certain plasma and serum components It is convenient therefore to consider the conversion of prothrombin as a reaction involving a number of such factors and to regard them as a prothrombin complex

THE ONE STAGE PROTHROMBIN TIME

Prothrombin activity may be measured by the single stage method of Quick and involves the addition of tissue thromboplastin to decalcified plasma with subsequent recalcification The time taken for a clot to appear is termed the prothrombin time and it is essential to control the reagents with normal plasmas The test may be reported as an index or a ratio for example, normal prothrombin time 15 seconds test plasma 45 seconds

Prothrombin Index $\frac{15}{45} \times 100 = 33\frac{1}{3}$ per cent efficiency

Ratio 45 15 = 3 1

The recommended method is to report the times of both test and normal plasmas in seconds but many authorities calculate a percentage of prothrombin from a dilution curve This is prepared by making a series of dilutions of normal citrated plasma in physiological saline and performing the test on each dilution By plotting prothrombin times against plasma percentages a curve (Fig 35) is

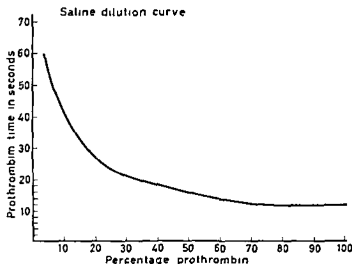


FIG 35 —Saline dilution curve (Thromboplastin Stayne)

obtained from which prothrombin percentages are read as a dilution of plasma This assumes that prothrombin is a pure substance acting without assistance but regarded as a complex it will be seen that allied factors are also diluted The ideal diluent for such curves has not yet been demonstrated The end point of the weaker dilu tions in this test is difficult to determine since fibrinogen is also diluted

The one stage prothrombin time actually measures the speed of prothrombin conversion and fibrinogen conversion Anti thrombins also take part prolonging the time by inactivation of thrombin The test therefore depends upon concentration of prothrombin the associated Factors V and VII, fibrinogen and possible anti-thrombins

TISSUE THROMBOPLASTINS

Preparations of thromboplastic activity obtained from tissue (lung brain etcetera) differ from that produced by shed blood Tissue thromboplastin needs calcium and Factors V and VII for rapid prothrombin conversion *Factor V is essential for efficient* thromboplastin generation by shed blood but after such thrombo plastin is produced neither Factor V nor VII is needed for pro thrombin conversion *In vivo* the importance of Factor VII in prothrombin conversion seems to be in injury where incomplete thromboplastins are released from the tissues Russell viper venom plus lecithin (lipoid factor) has thromboplastic activity but is not *complete since it does not possess Factor X (Stuart or Prower* factor) However knowing in what respects such thromboplastin preparations are deficient makes them useful tools in the investiga tion of haemorrhagic disorders

PROTHROMBIN CONSUMPTION

During coagulation of shed blood prothrombin is converted quantitatively into thrombin the process depending upon an efficient thromboplastin generation system Failure to generate efficient thromboplastin results in poor utilization of prothrombin which can then be demonstrated in the serum The test is good for screening purposes since it indicates an haemorrhagic disorder due to a deficiency of one or more of the factors necessary for thromboplastin generation All that is required is a one stage prothrombin time on both plasma and serum The result is expressed as an index the normal being from 0–40 per cent Standard conditions for the pro

between prothrombin and certain plasma and serum components It is convenient therefore to consider the conversion of prothrombin as a reaction involving a number of such factors and to regard them as a prothrombin complex

THE ONE STAGE PROTHROMBIN TIME

Prothrombin activity may be measured by the single stage method of Quick and involves the addition of tissue thromboplastin to decalcified plasma with subsequent recalcification The time taken for a clot to appear is termed the prothrombin time and it is essential to control the reagents with normal plasmas The test may be reported as an index or a ratio for example, normal prothrombin time 15 seconds test plasma 45 seconds

Prothrombin Index $\frac{15}{45} \times 100 = 33\frac{1}{3}$ per cent efficiency

Ratio 45 15 = 3 1

The recommended method is to report the times of both test and normal plasmas in seconds but many authorities calculate a per centage of prothrombin from a dilution curve This is prepared by making a series of dilutions of normal citrated plasma in physio logical saline and performing the test on each dilution By plotting prothrombin times against plasma percentages a curve (Fig 35) is

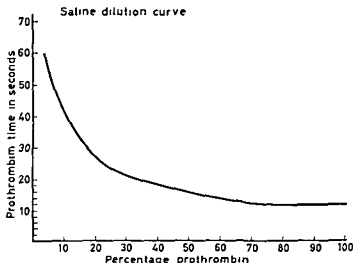


FIG 35—Saline dilution curve (Thrombo plastin Stayne)

INTRAVASCULAR THROMBOSIS

Venous or arterial thrombosis is not an uncommon post operative complication and may occur in a susceptible individual over middle age without surgical interference Modern treatment of the condition is by means of anticoagulants, such therapy being controlled by the laboratory to ensure that clotting factors are not depressed to dangerously low levels

Heparin, the physiological anti thrombin, is used at the start of treatment since its effect administered intravenously is very rapid Coumarin derivatives are slower and more variable in their effect but are given orally and possibly assist recanalization of the affected vessels Heparin therapy is controlled on the clotting time, and the administration of dicoumarol by the one stage prothrombin time which is very sensitive to the decreased concentration of Factor VII caused by the drug Overdosage with salicylates results in a prolonged one stage prothrombin time and it is interesting to note that dicoumarol can be denatured quantitatively to salicylic acid Salicylates plus dicoumarol are clinically dangerous, the danger of such drugs being haemorrhage In lung disease or congestive heart failure the prothrombin time is likely to lengthen very rapidly on small doses of dicoumarol and even without a partly healed wound or raw surface haemorrhage may occur into kidneys, bowel or brain The action of heparin can be reversed immediately by giving protamine sulphate but dicoumarol overdosage needs transfusion with fresh blood or plasma to check the haemorrhagic tendency Natural vitamin K_1 and its homologues also reverse the effect but they are much too slow in their action for emergency use

Toluidine blue inhibits the action of heparin so that the so called heparin effect that is prolongation of prothrombin time due to heparin can be nullified and the true effect of dicoumarol estimated Probably the best technique for the control of dicoumarol therapy is that of Owren in which the plasma is diluted to enhance small differences in clotting times and Factor V and fibrinogen supplied in large amounts by the addition of normal plasma adsorbed with barium sulphate

TRUE PROTHROMBIN

Prothrombin may be deficient in severe liver disease and a congenital absence of the factor has been described Newly born infants are deficient in prothrombin gross deficiency resulting in haemorrhagic disease of the new born This condition is treated

duction of serum must be followed rigidly and since serum is deficient in fibrinogen this must be provided in the test

FACTOR V DEFICIENCY

Factor V is the symbol used for the inactive precursor of a plasma component essential for blood thromboplastin The activated factor is termed Factor VI Factor V is the labile factor of Quick (pro thrombin component A) being destroyed on storage at 4 C for 14 days but it keeps much better at an acid *p*H and lower tempera tures The factor is produced in the liver and is reduced in severe hepatic disease An inherited or congenital deficiency has been described The one stage prothrombin time is abnormally pro longed and prothrombin consumption poor Treatment of normal plasma with inorganic adsorbents such as $BaSO_4$ $Ca_3(PO_4)_2$ or $Al(OH)_3$ results in a prothrombin free plasma which contains Factor V PTA Factor VIII and fibrinogen This product can be used as a source of Factor V and used to correct the prothrombin time thus confirming the diagnosis

FACTOR VII DEFICIENCY

Factor VII is a serum factor which is not used up during the clotting process A congenital deficiency has been described and production by the liver is depressed by carcinoma tuberculosis polycythaemia and severe sepsis An haemorrhagic disease of sheep and cattle caused by eating spoiled sweet clover is due to the presence in the foodstuff of dicoumarol a drug which depresses the formation of Factor VII and to a lesser extent prothrombin In Factor VII deficiency the one stage prothrombin time is prolonged but is corrected by the addition of normal serum to the test Prothrombin consumption in the disorder is normal

FACTOR X DEFICIENCY

It is convenient at this stage to consider deficiency of Factor X since the one stage prothrombin time is prolonged The component may be identical with Prower and Stuart factors which have been described as deficient in rare haemorrhagic disorders in families where there is some evidence of consanguinity The factor is differentiated from Factor VII by the fact that thromboplastin generation is impaired in its absence and the one stage prothrombin time greatly prolonged when Russell viper venom is used as a source of thromboplastin

from the dilution curve, the units having no real significance, being used only to compare with the normal From a typical curve (Fig 37) it can be seen that the prothrombin level can be estimated on the peak of the curve, that is, the greatest amount of thrombin formed or on the sum of the peaks, that is the total number of units By far the most accurate method however, is to estimate the area enclosed by the curve either by the laborious method of counting the squares or by using a planimeter

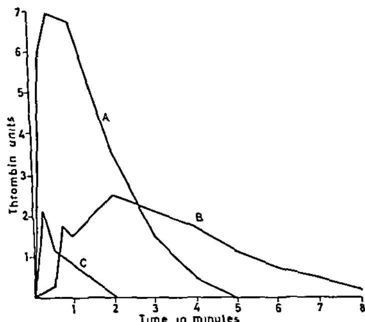


FIG 37—The two stage measurement of pro thrombin A Normal curve B Dindevan plasma C hypoprothrombinaemia (*By courtesy of Dr Rosemary Biggs and the Editor J Clin Path*)

DISORDERS OF THROMBOPLASTIN GENERATION

A deficiency of any of the factors necessary for efficient production of thromboplastin may produce an haemorrhagic disorder of lesser or greater severity Many of these disorders are inherited, some of them acquired and combined disorders are possible

Haemophilia

Haemophilia has been known from the earliest times but the term now is restricted to disorders in which there is a deficiency of anti

with vitamin K but overdosage has been reported as a cause of haemolytic anaemia

A true measurement of prothrombin is made by means of the two stage method of Biggs and Douglas in which all available prothrombin is converted to thrombin which is assayed by its action on fibrinogen Since prothrombin is converted quantitatively to thrombin the test also measures prothrombin It is assumed for the purposes of the test that the antithrombin strengths of test and normal plasma are similar

Two stage method

It is necessary in the two stage method to construct a reference curve derived from experiments with pure thrombin and fibrinogen Freshly prepared dilutions of thrombin topical (Parke Davis) are added to fibrinogen clotting times recorded and plotted against thrombin units The thrombin fibrinogen dilution curve (Fig 36)

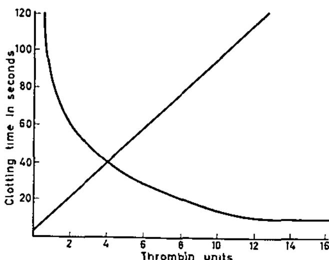


FIG 36—Thrombin fibrinogen dilution curve (*By courtesy of the author (Dr Rosemary Biggs) and publishers (Blackwell Oxford) of Prothrombin Deficiency*)

can be expressed as a straight line by plotting the clotting times against the reciprocals of the concentrations of thrombin In the test proper plasma is mixed with diluted brain thromboplastin and calcium chloride and the clotting mixture subsampled at intervals into fibrinogen The clotting times are converted to thrombin units

manner and the factor is concerned with plasma activation by con tact with glass There seems to be some relationship between PTA and Hageman factor in this respect

Haemorrhagic disorders and pregnancy

Haemorrhagic disorders associated with pregnancy have been shown to possess an antibody which has the effect of inhibiting or destroying anti haemophilic globulin Similar antibodies having the effect of a circulating anticoagulant have been produced in haemophilics by transfusion of fresh blood or the purified globulin, since this material being protein acts as an antigen Bridge anticoagulant is an anti Factor VIII substance present in the blood of haemophilics from birth It is relatively stable, whereas antihaemophilic globulin is very labile

Thromboplastin generation is disturbed by a qualitative defect of platelets in thrombasthaenia An abnormal thromboplastin genera tion may also appear in conditions where the platelets are increased beyond the normal numbers

CIRCULATING ANTICOAGULANTS

Many cases of plasma factor deficiencies can be shown to be accompanied by thromboplastin inhibitors which act as circulating anticoagulants The presence of circulating anticoagulants may be demonstrated by the thromboplastin generation test or by mixing tests on recalcified plasma

Heparin may be increased in amount in certain conditions particularly irradiation In such cases an haemorrhagic tendency may develop

CALCIUM CLOTTING TIME

Clotting factors involved in thromboplastin generation may be identified by mixing tests using the calcium clotting time which involves the recalcification of fresh citrated plasma The addition of plasma from Christmas disease corrects the calcium time of haemophilic plasma and the addition of haemophilic serum corrects the calcium time of Christmas disease plasma PTA deficiency is corrected with either serum or plasma from these conditions, tests using serum sometimes giving peculiar answers The normal calcium clotting time is 90–250 seconds but results are affected by platelet numbers and the time plasma is kept in contact with glass

THE THROMBOPLASTIN GENERATION TEST

The TGT literally consists of a series of one stage prothrombin

haemophilic globulin The disorder is transmitted as a sex linked characteristic for which there is a high mutation rate The inheritance is shown in Fig 38 It was originally assumed that a prolonged clotting time was a prime criterion of the disease, but some cases show genetic and medical evidence of the disease with a

F 1

	X	Y
$\underline{X}$	$\underline{X}X$	$\underline{X}Y$
X	XX	XY

F 2

	$\underline{X}$	Y
$\underline{X}$	$\underline{X}\underline{X}$	$\underline{X}Y$
X	$\underline{X}X$	XY

Fig 38 —The inheritance of haemophilia Two families are illustrated F 1 shows the possible progeny of the carrier female and normal male F 2 the carrier female and affected male the symbol X indicates the gene carried on the female (X) chromosome

clotting time normal or only slightly prolonged The recalcification time of plasma however and prothrombin consumption may be abnormal even when the clotting time is normal Such cases are termed mild haemophilia and breed true

Christmas factor deficiency

Christmas factor deficiency was first discovered when it was found that the clotting deficiency of haemophilic blood was corrected by the addition of serum from another patient with similar symptoms The factor is normally present in serum and is readily absorbed by inorganic absorbents Christmas disease and haemophilia have a ratio of 1 10 among bleeders in England The inheritance of the disorder is similar to that of haemophilia but there may be a difference between the penetrance of the genes since in the former disorder mildly affected females are more frequently encountered

PTA deficiency

Plasma thromboplastin antecedent (PTA) deficiency was first described by Rosenthal in 1953 It appears to be inherited by means of a dominant gene The factor is present in both serum and plasma is stable and is not absorbed by inorganic adsorbents

Hageman factor deficiency

The defect in Hageman factor deficiency does not result in an haemorrhagic diathesis The deficiency is inherited in a recessive

(5) Ampoule in 0 3 g amounts and seal in atmosphere of dry nitrogen

The dried brain may actually be kept in bulk in a glass stoppered bottle at room temperature for months without loss of potency

Reagent (*b*) Reconstituted extract
Suspend 0 3 ml dried brain in 5 0 ml normal saline and activate at 37 C for 30 minutes

Reagent (*c*) M/40 calcium chloride

Reagent (*d*) Citrated plasma

(1) Collect venous blood into 3 8 per cent sodium citrate in the proportions of 9 parts of blood to 1 of anticoagulant
(2) Repeat the procedure with at least two normal bloods

Test proper

(1) Place 0 1 ml of plasma in 3 inches × ⅜ inch test tube in the water bath at 37 C together with a quantity of brain thromboplastin and M/40 $CaCl_2$
(2) Allow the reagents to warm to 37 C and add 0 1 ml saline brain suspension to the plasma
(3) Add 0 1 ml $CaCl_2$ to mixture and start stop watch Note time taken for clot to form
(4) Repeat the procedure with normal plasmas Normal 12–15 seconds

N B It is useful to have a strong light and a black background to facilitate reading

SALINE DILUTION CURVE

(1) Dilute fresh citrated plasma from a normal subject to 90 80 70 60 50 40 30 20 and 10 per cent with normal physiological saline
(2) Perform one stage prothrombin time on each dilution
(3) Plot times in seconds against plasma percentages

CORRECTION OF PROTHROMBIN TIME FOR FACTOR V AND VII DEFICIENCIES

Reagent (*a*) Prothrombin time reagents

Reagent (*b*) Normal serum

Reagent (*c*) Normal adsorbed plasma (*see* below)

PRESUMPTIVE TEST FOR FACTOR V DEFICIENCY

(1) Perform one stage prothrombin time on patient s plasma
(2) Repeat the procedure on a mixture of 9 parts of patient s plasma and 1 part of normal adsorbed plasma

If the prothrombin time is substantially shortened *by the addition* of normal adsorbed plasma a deficiency of Factor V is proven

times but instead of using tissue thromboplastin that produced by blood is used and the essential components from the patient substituted one by one for the normal to decide which is actually deficient in activity Normal platelets, serum and absorbed plasma are mixed together with calcium and the rate of thromboplastin generation measured by subsampling at intervals into a substrate of platelet free normal plasma as a source of prothrombin and fibrinogen This constitutes a normal run and comparison is made by substituting in turn reagents from the patient Since preliminary investigations will have demonstrated any deficiency of Factors V or VII this test is used to reveal deficiencies of Factors VIII IX and platelet factors and also the presence of circulating anticoagulants

Preparation of reagents for the TGT is tedious but a screening test has been evolved (Hicks and Pitney 1957) which utilizes whole plasma from the patient and in some respects is even more sensitive than the standard thromboplastin generation test To obviate the preparation of platelet suspensions a lipoid extract of brain thromboplastin may be employed with the same reaction but only if a platelet factor is not at fault

ASSAY OF AHG

The thromboplastin generation test can be modified to assay the amount of AHG present in samples of normal and haemophilic plasma The amounts of thromboplastin generated by a normal system grossly deficient in AHG to which is added dilutions of the test and normal plasmas may be compared assuming the standard normal plasma to contain 100 per cent AHG In haemophilics the range is from 0 to 25 per cent

TECHNIQUE

The following are the details of test techniques appertaining to the prothrombin complex

ONE STAGE PROTHROMBIN TIME (QUICK)

Reagent (*a*) Dried brain powder

(1) Collect fresh human brain from the post mortem room and remove all superficial vessels and membranes
(2) Macerate the brain with 3–4 times its volume of acetone
(3) Pour off acetone and replace with fresh repeating the process 4 times
(4) Dry the crumbly material on a suction filter

(3) Repeat with plasma from the patient
(4) If plasma clotting time from patient is increased above the normal replace the saline addition with 0 1 ml toluidine blue or protamine sulphate

If the clotting time of plasma returns to normal on addition of toluidine blue or protamine sulphate then the anticoagulant is heparin in nature

OWREN S PROTHROMBIN AND PROCONVERTEN METHOD OF CONTROLLING THERAPY WITH DICOUMAROL

Reagent (a) Fresh brain extract
(1) Obtain human brain from the post mortem room and free it from superficial vessels and membranes
(2) Macerate the whole brain for 2 minutes in a blender with 1 500 ml of normal saline heated to 37° C
(3) Centrifuge the emulsion for 25 minutes at 2 000 r p m and discard the sediment
(4) Use the supernatant in the one stage prothrombin time of normal plasma diluting the thromboplastin 1/2 1/3 and 1/5 with saline
(5) Select the dilution which gives the shortest clotting time and dilute it approximately with saline
(6) Add 10 per cent of Owren s buffer and dispense in small amounts Store frozen solid at − 20 C

Reagent (b) Owren s buffer

Sodium diethylbarbiturate	11 75 g
Sodium chloride (AR)	14 67 g
N/10 hydrochloric acid	430 ml
Distilled water	1 570 ml

The final solution should be *p*H 7 35

Reagent (c) Barium sulphate
(1) Suspend 1 lb of x ray $BaSO_4$ in 4 000 ml of 0 005 M trisodium citrate ($Na_3C_6H_5O^7$ $2H_2O$) and allow to stand overnight
(2) Draw off supernatant and discard Repeat the procedure with a further 4 000 ml of citrate solution and allow to settle
(3) Collect the sediment on strong filter paper and dry in the oven at 100 C
(4) Powder the dried material thoroughly

Reagent (d) Adsorbed ox plasma
(1) Collect ox blood into 2 5 per cent potassium oxalate in the proportion of 1 part of oxalate to 9 parts of blood
(2) Centrifuge and take off plasma
(3) Treat the plasma with $BaSO_4$, allowing 2g $BaSO_4$ for each 100 ml of plasma
(4) Stir the mixture mechanically for 30 minutes at room temperature

PRESUMPTIVE TEST FOR FACTOR VII DEFICIENCY

(1) Perform one stage prothrombin time on patient s plasma
(2) Repeat the procedure on a mixture of 9 parts of patient s plasma and 1 part of normal serum

If the prothrombin time is substantially shortened by the addition of normal serum a deficiency of Factor VII is proven

PROTHROMBIN CONSUMPTION INDEX

Reagent (a) Prothrombin time reagents

Reagent (b) Fibrinogen 100–200 mg per cent

Reagent (c) Citrated plasma

Reagent (d) Serum

(1) Collect venous blood into clean dry tubes 3 inches × ⅜-inch in 1 0 ml amounts
(2) Allow the blood to clot at 37 C and exactly 1 hour after coagulation has taken place centrifuge and remove serum

Test

(1) Place quantities of brain thromboplastin citrated plasma fibrinogen and calcium chloride in water bath at 37° C for a few minutes to warm up
(2) In a clean 3 inches × ⅜ inch test tube place 0 1 ml brain thromboplastin 0 1 ml plasma and after the addition of 0 1 ml. M/40 $CaCl_2$ start a stop watch A clot will form rapidly and must be removed with an applicator
(3) Exactly one minute after the addition of calcium chloride rapidly add 0 2 ml fibrinogen
(4) Repeat the procedure using patient s serum instead of plasma No clot forms on the addition of $CaCl_2$ because of absence of fibrinogen

$$\text{P C I} = \frac{\text{Plasma clotting time in seconds}}{\text{Serum clotting time in seconds}} \times 100$$

Normal 0–40 per cent

HEPARIN ACTIVITY

Reagent (a) Thrombin topical (Roche)
Reagent (b) 0 05 per cent toluidine blue in normal physiological saline
Reagent (c) 0 25 per cent protamine sulphate in saline

Method

(1) Prepare two dilutions of thrombin solution which will clot normal plasma in 8 seconds and 20 seconds respectively
(2) Add 0 1 ml of each thrombin dilution to 0 1 ml. of normal plasma plus 0 1 ml saline Note clotting times

(3) Repeat with plasma from the patient.
(4) If plasma clotting time from patient is increased above the normal replace the saline addition with 0 1 ml toluidine blue or protamine sulphate

If the clotting time of plasma returns to normal on addition of toluidine blue or protamine sulphate then the anticoagulant is heparin in nature

OWREN S PROTHROMBIN AND PROCONVERTEN METHOD OF CONTROLLING THERAPY WITH DICOUMAROL

Reagent (*a*) Fresh brain extract

(1) Obtain human brain from the post mortem room and free it from superficial vessels and membranes
(2) Macerate the whole brain for 2 minutes in a blender with 1,500 ml of normal saline heated to 37° C
(3) Centrifuge the emulsion for 25 minutes at 2 000 r p m and discard the sediment
(4) Use the supernatant in the one stage prothrombin time of normal plasma diluting the thromboplastin 1/2 1/3 and 1/5 with saline
(5) Select the dilution which gives the shortest clotting time and dilute it approximately with saline
(6) Add 10 per cent of Owren s buffer and dispense in small amounts Store frozen solid at − 20° C

Reagent (*b*) Owren s buffer

Sodium diethylbarbiturate	11 75 g
Sodium chloride (AR)	14 67 g
N/10 hydrochloric acid	430 ml
Distilled water	1 570 ml

The final solution should be *p*H 7 35

Reagent (*c*) Barium sulphate

(1) Suspend 1 lb of x ray $BaSO_4$ in 4 000 ml of 0 005 M trisodium citrate ($Na_3C_6H_5O_7 \; 2H_2O$) and allow to stand overnight
(2) Draw off supernatant and discard Repeat the procedure with a further 4 000 ml of citrate solution and allow to settle
(3) Collect the sediment on strong filter paper and dry in the oven at 100 C
(4) Powder the dried material thoroughly

Reagent (*d*) Adsorbed ox plasma

(1) Collect ox blood into 2 5 per cent potassium oxalate in the proportion of 1 part of oxalate to 9 parts of blood
(2) Centrifuge and take off plasma
(3) Treat the plasma with $BaSO_4$ allowing 2g $BaSO_4$ for each 100 ml of plasma
(4) Stir the mixture mechanically for 30 minutes at room temperature

PRESUMPTIVE TEST FOR FACTOR VII DEFICIENCY

(1) Perform one stage prothrombin time on patient s plasma
(2) Repeat the procedure on a mixture of 9 parts of patient s plasma and 1 part of normal serum

If the prothrombin time is substantially shortened by the addition of normal serum a deficiency of Factor VII is proven

PROTHROMBIN CONSUMPTION INDEX

Reagent (*a*) Prothrombin time reagents

Reagent (*b*) Fibrinogen 100–200 mg per cent

Reagent (*c*) Citrated plasma

Reagent (*d*) Serum

(1) Collect venous blood into clean dry tubes 3 inches × ⅜ inch in 1 0 ml amounts
(2) Allow the blood to clot at 37 C and exactly 1 hour after coagulation has taken place centrifuge and remove serum

Test

(1) Place quantities of brain thromboplastin citrated plasma fibrinogen and calcium chloride in water bath at 37° C for a few minutes to warm up
(2) In a clean 3 inches × ⅜ inch test tube place 0 1 ml brain thromboplastin 0 1 ml plasma and after the addition of 0 1 ml M/40 $CaCl_2$ start a stop-watch A clot will form rapidly and must be removed with an applicator
(3) Exactly one minute after the addition of calcium chloride rapidly add 0 2 ml fibrinogen
(4) Repeat the procedure using patient s serum instead of plasma No clot forms on the addition of $CaCl_2$ because of absence of fibrinogen

$$\text{P C I} = \frac{\text{Plasma clotting time in seconds}}{\text{Serum clotting time in seconds}} \times 100$$

Normal 0–40 per cent

HEPARIN ACTIVITY

Reagent (*a*) Thrombin topical (Roche)

Reagent (*b*) 0 05 per cent toluidine blue in normal physiological saline

Reagent (*c*) 0 25 per cent protamine sulphate in saline

Method

(1) Prepare two dilutions of thrombin solution which will clot normal plasma in 8 seconds and 20 seconds respectively
(2) Add 0 1 ml of each thrombin dilution to 0 1 ml of normal plasma plus 0 1 ml saline Note clotting times

(5) Repeat the tests using $CaCl_2$ concentrations of M/10 M/20 and M/30 selecting as optimal concentration that which gives minimum clotting times

Interpretation of test

Read plasma concentration from clotting times on dilution curve Therapeutic range using dicoumarol lies between 10 and 20 per cent of normal

THROMBIN FIBRINOGEN DILUTION CURVE

(1) Dilute thrombin to contain 20, 15 10 8 5 3 2 1 and 0 5 units of thrombin per ml
(2) Place a series of tubes containing 0 4 ml amounts of fibrinogen in the water bath at 37 C
(3) Add 0 1 ml of thrombin solutions to the fibrinogen tubes and record clotting times
(4) Plot clotting times against thrombin concentrations

N B Thrombin solutions must be freshly prepared

TWO STAGE PROTHROMBIN

(1) Dilute brain thromboplastin with saline to give a one stage pro thrombin time with normal plasma of 20–25 seconds
(2) Mix 0 4 ml normal citrated plasma with 0 4 ml diluted brain thromboplastin
(3) Add 0 4 ml M/40 $CaCl_2$ and start stop watch
(4) At intervals of 30 seconds 1 2 3 4 5 and 6 minutes subsample 0 1 ml into 0 4 ml amounts of fibrinogen and record clotting times
(5) Continue test until clotting time exceeds 180 seconds and repeat on patient s plasma
(6) Interpret clotting times in terms of thrombin units from thrombin fibrinogen dilution curve and draw a curve relating thrombin units to incubation time
(7) Compute the areas enclosed by the two curves and express result as percentage of the normal

N B When clotting occurs in the incubation mixture remove it with a wooden applicator

RECALCIFICATION TIME (CALCIUM CLOTTING TIME)'

(1) To 0 1 ml citrated plasma add 0 1 ml 0 85 per cent saline and place in water bath at 37 C
(2) Add 0 1 ml M/40 $CaCl_2$ and start stop watch Note time taken for clot to appear

Normal by this method 90–250 seconds

(5) Centrifuge to remove the barium sulphate
(6) Test the adsorbed plasma by (*a*) and (*b*)

(*a*) Mix 0 1 ml adsorbed plasma 0 1 ml saline 0 1 ml brain extract 0 1 ml M/20 $CaCl_2$	}	Clotting time should exceed 30 minutes
(*b*) Mix 0 1 ml adsorbed plasma 0 1 ml 1/10 dilution of fresh normal serum 0 1 ml brain extract 0 1 ml M/20 $CaCl_2$	}	Clotting time should exceed 90 seconds

(7) Store the adsorbed plasma frozen solid at − 20 C

Reagent (*e*) Diluting solutions

(*a*) 3 13 per cent trisodium citrate	100 ml
0 9 per cent sodium chloride	600 ml
(*b*) Owren buffer	200 ml
0 75 per cent trisodium citrate	200 ml
0 9 per cent sodium chloride	600 ml

Reagent (*f*) Anticoagulant for collection of blood

3 13 per cent trisodium citrate	250 ml
Heparin	25 mg
Sodium merthiolate	25 mg

The heparin prevents alterations in clotting time of samples sent by post

Collect 9 parts of blood to 1 of anticoagulant

Reagent (*g*) Normal control plasma
(1) Pool plasma from ten donors and mix
(2) Store in small amounts frozen solid at − 20 C

Test proper

(1) Dilute the test plasma 1 10 with diluting fluid (*a*)
(2) Mix 0 1 ml diluted plasma with 0 1 ml $BaSO_4$ treated ox plasma 0 1 ml brain extract and 0 1 ml $CaCl_2$ of optimal concentration (*see* below) Start stop-watch on addition of calcium chloride Normal clotting time 35–45 seconds

DILUTION CURVE

(1) Make dilutions of normal pooled plasma 1 2 1 4 1 8 and 1 16 using diluting fluid (*a*)
(2) Further dilute these dilutions and undiluted plasma 1 10 using diluting fluid (*b*) to give final dilutions of 1 10 1 20 1 40 1 80 and 1 160 the dilutions having a constant ionic strength
(3) Carry out the test in duplicate on each dilution
(4) Plot the clotting times against plasma concentration on double logarithmic graph paper A straight line should be obtained

(2) Centrifuge at 2 000 r p m for 15 minutes and decant the treated plasma
(3) Test the plasma by the one stage prothrombin method Time should exceed 1 minute but be less than 4 minutes

Reagent (*c*) Citrated plasma
(1) Mix 9 parts of venous blood with 1 part of 3 8 per cent sodium citrate
(2) Separate plasma by centrifuging at 2 000 r p m for 15 minutes

Reagent (*d*) Platelet suspension
(1) Using a siliconed syringe collect 9 ml of venous blood into 1 ml of 3 8 per cent sodium citrate in a siliconed tube
(2) Centrifuge at 1 000 r p m for 10 minutes and remove plasma with a siliconed pipette into another siliconed tube
(3) Centrifuge at 3 000 r p m for 30 minutes and decant the high spun platelet free plasma This constitutes substrate plasma
(4) Wash the platelets twice with saline breaking up the clump with an applicator
(5) Resuspend in saline to one third of the original plasma volume The suspension maintains its activity 1–6 weeks if kept frozen solid at -20 C

Reagent (*e*) Serum
(1) Collect 3–4 ml venous blood into small tube containing 4 small glass beads
(2) Incubate at 37° C to clot inverting the tube several times just before clotting occurs Leave at 37° C for 3 hours
(3) Allow to stand overnight and centrifuge

Reagent (*f*) Owren veronal buffer *p*H 7 35

Test proper

(1) Add 0 1 ml normal high spun plasma to each of six 3 inches × ⅜ inch tubes in the water bath at 37° C
(2) In another tube place 0 2 ml of adsorbed normal plasma diluted 1 5 with buffer 0 2 ml of serum diluted 1 10 with buffer and 0 2 ml platelet suspension and allow the mixture to warm
(3) Add 0 2 ml M/40 $CaCl_2$ and start stop watch
(4) At one minute intervals subsample 0 1 ml of the mixture together with 0 1 ml M/40 $CaCl_2$ from different pipettes into fibrinogen and note clotting times

N B The mixture clots within 2–3 minutes and the clot must be removed with an applicator

(5) Repeat substituting in turn patient s platelets serum and plasma

MIXING TESTS WITH RECALCIFICATION TIME

(1) Perform test as before but replace saline with normal plasma fibrinogen fraction haemophilic plasma Christmas disease plasma and normal serum The clotting time of haemophilics is corrected by normal or Christmas disease plasma while clotting time of Christmas disease is corrected by normal or haemophilic serum PTA deficiency clotting time is corrected either with serum or plasma

THROMBOPLASTIN GENERATION TEST (Table VIII)

Reagent (*a*) Aluminium hydroxide gel

(1) Dissolve 22 g of ammonium sulphate in 600 ml of distilled water and bring to 63° C
(2) Add 100 ml ammonia solution (S G 0 88 diluted one half) and bring temperature rapidly to 58 C
(3) Stir the mixture vigorously and pour rapidly in one lot into a solu tion of 76 7 g ammonium alum in 1 000 ml distilled water at a temperature of 58 C The temperature rises to 61 C
(4) Stir the mixture for 10 minutes keeping the temperature above 58 C and then centrifuge

TABLE VIII

TYPICAL RESULTS OBTAINED WITH THE THROMBOPLASTIN GENERATION TEST

Disorder	*Platelets*	*Adsorbed plasma*	*Serum*	*Clotting time seconds after incubation*					
				1 *min*	2 *min*	3 *min*	4 *min*	5 *min*	6 *min*
Normal	Normal	Normal	Normal	30	18	10	8	8	9
Haemophilia	Normal	Normal	Patient	18	11	8	8	7	9
	Normal	Patient	Normal	44	40	30	28	28	29
Christmas disease	Normal	Normal	Patient	36	30	26	20	3	23
	Normal	Patient	Normal	34	18	12	8	9	9
Circulating anticoagulant	Normal	Normal	Patient	40	20	16	16	17	16
	Normal	Patient	Normal	70	58	57	57	42	42

(5) Wash the precipitate five times with 1 500 ml distilled water separating each time by centrifuging Add 0 44 ml of ammonia (S G 0 88 diluted one half) to the first washing water and 0 88 ml to the second
(6) Suspend the precipitate in the least amount of water required to make a gelatinous suspension which can be pipetted

Reagent (*b*) Alumina treated plasma

(1) Add 0 05–0 1 ml aluminium hydroxide suspension to each ml of plasma and incubate at 37 C for 1–3 minutes

(2) Centrifuge immediately at 3 000 r p m for 15 minutes
(3) Store plasma frozen solid at -20° C in small volumes
(4) Test by one stage method for Factor V activity It should not be deficient in this factor
(5) Before use adsorb with 0 1 ml alumina gel

Reagent (*b*) Normal serum
(1) In a small tube containing 4 glass beads place 3–4 ml of blood and allow to stand Invert the tube several times just before the blood clots
(2) After the blood has clotted allow it to stand at 37 C for 24 hours
(3) Separate the serum and store frozen at -20 C
(4) Three to four hours before the test dilute 1 ml of serum with 9 ml of veronal buffer and keep diluted reagent at 4 C before the test

Reagent (*c*) Platelet suspension prepared as for the thromboplastin generation test

Reagent (*d*) Normal plasma
Adsorb normal citrated plasma with alumina gel and dilute in veronal buffer to give concentrations of 1 20 to 1 2 000 The 1 20 dilution is the standard 100 per cent AHG

Reagent (*e*) Patient s plasma
Adsorb patient s plasma with alumina gel and dilute with veronal buffer to give dilutions of 1 5 1 10 1 20 1 40 and 1 80

Test proper

(1) To a small tube at 37° C add 0 1 ml of 1 5 adsorbed haemophilic plasma 0 1 ml of 1 10 normal serum 0 1 ml platelet suspension and 0 1 ml of one of the dilutions of adsorbed normal plasma Allow to warm to 37 C
(2) Add 0 1 ml warm 0 025 M $CaCl_2$ and start stop watch
(3) After 4 minutes incubation subsample 0 1 ml together with 0 1 ml of 0 025 M $CaCl_2$ to 0 1 high spun normal plasma
(4) Plot the clotting times against the corresponding percentage of AHG (dilution of normal plasma)
(5) Assay the test plasma using three dilutions of plasma If AHG is grossly deficient use the 1 5 1 10 and 1 20 dilutions if not so severe the 1 20 1 40 and 1 80 dilutions
(6) Read off AHG concentration from clotting times on the calibration curve and average

DEMONSTRATION OF CIRCULATING ANTICOAGULANT ACTIVITY

(1) At the beginning of the thromboplastin generation test place equal volumes of normal absorbed plasma and patient s adsorbed plasma in water bath at 37 C Leave at least 15 minutes

(2) Use incubated plasma in test in usual way

Plasma anticoagulant activity will be shown by even marked abnor mal thromboplastin generation

SCREENING TEST (HICKS AND PITNEY)

Reagent (*a*) Citrated plasma obtained by centrifuging at 3 000 r p m for 10 minutes

Reagent (*b*) Platelet substitute (Bell and Alton)

(1) Treat 1 g of brain thromboplastin with two successive 20 ml amounts of acetone and dry the residue

(2) Add 20 ml chloroform to the dried powder and extract for 2 hours at room temperature on the suspension mixer

(3) Filter and dry off chloroform extract in a flat dish

(4) Before the preparation is completely dry scrape off and suspend in 10 ml of saline

(5) Make dilutions 1 50 1 100 1 150 and 1 200 and test with normal plasma Use dilution with shortest clotting time

This reagent keeps very well

Reagent (*c*) Substrate plasma

Collect normal venous blood into 3 8 per cent sodium citrate and centrifuge at 3 000 r p m for 10 minutes

Reagent (*d*) Owren veronal buffer *p*H 7 35

Method

(1) Dilute patient s plasma with 9 parts of veronal buffer

(2) Add 0 5 ml diluted plasma and 0 5 ml platelet substitute to a 3 inches × ⅜ inch tube in a water bath at 37 C

(3) Add 0 5 ml of previously warmed calcium chloride and start stop watch

(4) At one minute intervals subsample 0 1 ml amounts together with 0 1 ml M/40 $CaCl_2$ into 0 1 ml amounts of substrate plasma

The pattern of results is similar to the thromboplastin generation test proper minimum clotting times being produced in 3-5 minutes varying between 6 5 and 11 seconds

ASSAY OF ANTI HAEMOPHILIC GLOBULIN (PITNEY)

Reagent (*a*) Haemophilic plasma

(1) Obtain venous blood from a severely affected haemophiliac and add to one tenth volume of 3 8 per cent sodium citrate

bacteria viruses and protozoa, collagen disorders, poisoning and irradiation, often with no obvious reason, and with more reason in haemopoietic disorders It may be that infections involving the spleen (such as malaria, kala azar, and so on) produce hypersplenism resulting in leucopenia Infectious hepatitis, portal cirrhosis of the liver and those cases of glandular fever with liver damage may show a leucopenia again for reasons which are obscure, but it is probable that the leucopenia of anaphylactoid shock is due to sequestration of leucocytes in the internal organs Irradiation and certain drugs—particularly the nitrogen mustards—act as mitotic poisons preventing cell division at some stage Such treatments usually act in the beginning more severely on cells of the lymphocyte series

Drugs causing maturation defects

Drugs which act on granulocyte precursors causing maturation defects include thiouracil, amidopyrine sulphonamides and organic arsenicals A temporary agranulocytosis may be produced and if treatment is continued other marrow elements may be affected with subsequent aplasia Differentiation between the agranulocytosis of a maturation defect and that of hypersplenism is made on bone marrow appearances

Other characteristic variations

The condition termed cyclical neutropenia is characterized by a reduction in circulating neutrophils at regular intervals The total white cell count may not drop too dramatically during relapses and in such cases atypical monocytes may appear to replace the absent neutrophils Splenectomy has no curative effect in these cases

Antibodies active against leucocytes can sometimes be demon strated in agranulocytosis particularly where there is a history of exposure to drugs An antigenic complex of drug and leucocyte is formed which in contact with antibody forming tissue causes the production of agglutinins or lysins The leucocytes become loaded with antibody and in the presence of complement are clumped and destroyed

An increase in the number of circulating leucocytes above 12 000 per c mm is termed leucocytosis and may be due to an increase in any one or more of the leucocyte elements Infections with pyogenic organisms usually produce a neutrophil leucocytosis providing the pus has a free outlet Should the pus be locked up the total count may not be greatly raised but a left shift can often be demonstrated by a nuclear lobe count Injury produces a neutrophil leucocytosis

CHAPTER 10

DISORDERS OF LEUCOPOIESIS

INTRODUCTION

THE LEUCOCYTES are concerned with body defence and it has been suggested that they are not really blood cells but merely use the blood stream to get to that part of the body where they are needed The fairly constant numbers and ratio of leucocytes in the circulation is an indication of the healthy controlled production of these cells Many leucocytes remain sequestrated in the internal organs where they act as a strategic reserve, being constantly replaced as the cells become senile In health the release of leucocytes from the marrow is governed to a large extent by the endocrine system Over activity of the control system such as that produced in hypersplenism can result in a low white cell count in spite of a hyperplastic marrow Cortical stimulation of the normal adrenal causes a decrease in circulating eosinophils, a phenomenon of value in the diagnosis of Addison s disease since the administration of ACTH has no such effect in this condition (*see* Chapter 3) Although the leucocytes are produced in different sites their production may be regarded as a tissue like the erythron and is known as the leucon

PHYSIOLOGICAL VARIATIONS

Individual normal white cell counts vary from 4–12 000 per c mm Strenuous exercise may release sequestrated cells from the spleen, a process which can also be initiated by the injection of adrenalin A daily rhythmic fluctuation of leucocyte numbers occurs and together with a post prandial rise provides good reason why comparative *counts should be performed at the same hour of each day* Normally eosinophil values are lower in the morning than in the evening

VARIATIONS IN DISEASE

A reduction in numbers of circulating leucocytes is termed leucopenia and in most cases is due to reduction in neutrophils This is then termed neutropenia the rarer cases of lymphocyte reduction being termed lymphopenia Leucopenia occurs in infections due to

the condition being termed leukaemia or leucosis Sometimes the white-cell count is within normal limits or even lower than normal, the abnormal cells remaining locked in the marrow This is termed aleukaemic leukaemia and may terminate as a frank leukaemia Since the leukaemias are disorders of the reticulo endothelial system they are classified as reticuloses and grouped as medullary reticu loses Although the stimulus which seems to initiate the leukaemic process can be infection drugs or irradiation the mode of action is unknown An inherited predisposition has been described and a viral cause of leukaemia in fowls proved but the exact aetiology of leukaemia is a mystery

Chronic myeloid leukaemia

In chronic myeloid leukaemia the white cell count ranges from 100 000 to 300 000 or more with a marked left shift The differential leucocyte count may show as much as 30 per cent of myelocytes with a few pro myelocytes and myeloblasts There is a progressive anaemia and thrombocytopenia the blood showing anisocytosis polychromasia basophilic stippling and a varying number of normoblasts The marrow shows a great preponderance of myeloid pre cursors with polyploid cells and mitoses The serum B_1 level in this condition is very high sometimes being fifteen times the normal

Acute myeloblastic leukaemia

In the acute form of myeloid leukaemia total white cell count ranges from 5 000–50 000 per c mm Most of the circulating cells are myeloblasts which may present difficulty in differentiation from other blast cells Micro myeloblasts particularly may resemble lymphocytes to the inexperienced since the nucleoli may not be very obvious Feulgen s reaction demonstrates the nucleoli very well Acute myeloid leukaemia is characterized by haemorrhage due to capillary damage and thrombocytopenia contributing to an anaemia which rapidly becomes severe The marrow is hyperplastic, most of the myeloid series being myeloblasts Red cell production is depressed and megakaryocytes often completely absent The B_{12} level is variable in this condition

Eosinophilic neutrophilic and basophilic leukaemias

A chronic eosinophilic leukaemia has been described with a pro gressive anaemia and thrombocytopenia and a total white cell count of less than 100 000 per c mm the majority of the cells being mature eosinophils The bone marrow is said to be hyperplastic with a pro-

haemorrhage contributing to this effect Burns produce a neutrophil leucocytosis roughly proportional to the degree of burning Certain poisons including carbon monoxide, some anaesthetics salicylates digitalis and benzol derivatives may produce a neutrophil leucocytosis but their effect is not predictable

A true lymphocytosis may occur in infants in response to infections which in the adult produce a neutrophil leucocytosis Whooping cough produces an invariable lymphocytosis, and many virus diseases including mumps measles smallpox and chickenpox may also exhibit this sign Over exposure to solar radiation can produce a lymphocytosis even accompanied by lymphadenopathy

Eosinophilia of more than 400 per c mm is regarded as significant Allergic and skin disorders worm infestations Hodgkin s disease Loeffler s syndrome irradiation and some poisons may produce an eosinophilia Trichinosis and filariasis produce a marked eosinophilia of as much as 70–80 per cent of all circulating leucocytes

Basophilia of over 200 per c mm is regarded as significant but the appearance is not often seen except in some true blood disorders

Monocytosis above 500 per c mm is found in some virus infections tuberculosis malaria typhoid and sometimes in cyclical neutropenia Some poisons also may produce this appearance

Plasma cells are not normally seen in the peripheral blood but are found in measles German measles and myelomatosis Since they are concerned with antibody production the presence of large numbers of plasma cells is associated with an increased serum globulin

MATURATION ANOMALIES

Alder s granulation anomaly is associated with coarse and plentiful granulation of neutrophils eosinophils basophils and lymphocytes There is no definite connexion with disease, but the phenomenon is seen in cases of gargoylism

Hegglin s inherited anomaly of maturation is associated with megakaryocyte defects but is also characterized by the presence of Dohle bodies in the neutrophils

THE LEUKAEMIAS

The leucocytes are derived from persisting mesenchyme and under normal circumstances production is strictly controlled Pathological stimuli however applied to the mesenchyme result in a neoplastic type of proliferation of one or other of the white cell elements The number of circulating leucocytes may rise to extremely high levels

terized by haemorrhage and a rapidly progressive anaemia The total leucocyte count varies from 10 000–500 000 per c mm but is usually 50 000–70 000 per c mm The predominant cell is the early lymphocyte which may comprise 90 per cent or more of the total true lymphoblasts are rare The blood film shows anisocytosis poikilocytosis polychromasia with numerous normoblasts Platelets may be entirely absent from the peripheral blood The marrow consists almost entirely of leukaemic cells

The aleukaemic form of lymphatic leukaemia is often difficult to diagnose In the medullary type the spleen and lymph nodes are apparently not involved but marrow puncture reveals lymphocytic infiltration Where lymph nodes are involved a biopsy may be inconclusive since the picture closely resembles that of lymphosarcoma in which disease the blood may show a typical leukaemic picture

Monocytic leukaemia

Two types of leukaemia are recognized as involving monocytes A purely monocytic type arising from the reticulo endothelial system is known as the Schilling type In this type the cell count is not diagnostic but anaemia haemorrhage and oral infection with gum hypertrophy are commonly seen Most of the cells are monocytes and their precursors but myelocytes may be present in the blood A rapidly progressive anaemia and thrombocytopenia quickly terminates the disease In the Naegeli type the leukaemic cell is derived from myeloid tissue This so called mixed leukaemia may present difficulties in diagnosis The marrow in both types of the disease shows monocytes and their precursors the Schilling type tending to show more reticulum cells than the Naegeli type An aleukaemic form of monocytic leukaemia has been described

Plasma cell leukaemia

A leukaemic process involving plasma cells is a rarity which probably does not exist as a clinical entity Although a described case had a total white cell count of 60 000 per c mm many of them being plasma cells and was associated with deposits of plasma cells in spleen lymph nodes and marrow it would seem that the cases should be classified as myelomatosis

Myeloma

Myelomatosis is a condition of single or multiple reddish tumours of the marrow The tumours consist of myeloma cells which have morphological characteristics in common with the plasma cell series

gressive left shift of the eosinophils A neutrophilic and a basophilic leukaemia have also been described but the evidence would seem capable of other interpretation

Chloroma

Sometimes in children a myeloid leukaemia blood picture may be associated with greenish tumours of the bone, the tumours consisting of myeloblastic cells The green colour fades on exposure to air but can be returned to the tumour by the addition of reducing agents such as sodium hydrosulphite to the mounting medium The pigment is a derivative of choleglobin one of the intermediate products between haemoglobin and bilirubin The blood and marrow picture is usually that of acute myeloblastic leukaemia

Aleukaemic leukaemia

Both acute and chronic leukaemia can present with a normal or low white cell count the condition being termed aleukaemic leukaemia of myeloid type A progressive anaemia and thrombocytopenia may cause the disease to be misdiagnosed as an aplastic anaemia but marrow puncture reveals the true diagnosis A useful procedure is to concentrate the white cells by making films from the buffy coat In this way small numbers of abnormal cells may be demonstrated

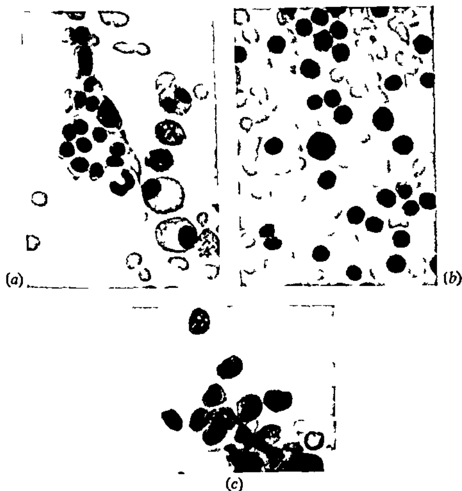
Chronic lymphatic leukaemia

Chronic lymphatic leukaemia is characterized by a slower developing anaemia than myeloid leukaemia and the predominant cell is a medium sized lymphocyte comprising 90 per cent of a total count of 20–100 000 per c mm The count may be even higher than the latter figure Owing to the fragile nature of the cells smudge or smear forms are common and there is a marked degree of thrombocytopenia In the terminal stages the blood may become flooded with lymphoblasts The marrow is infiltrated with lymphocytes the myeloid tissue depressed and there is some erythroid hyperplasia erythropoietic tissue being affected later than in myeloid leukaemia Clusters of lymphocytes seen in the marrow are an early sign of this disorder A rarer type of chronic lymphatic leukaemia shows the same blood and marrow picture but the predominant cell is a large lymphocyte

Acute lymphatic leukaemia

Acute lymphatic leukaemia is the commonest of the acute leukaemias It occurs in children and young adults and is charac

PLATE II



(*a*) Bone marrow—myelomatosis (*b*) Peripheral blood—lymphatic leukaemia
(*c*) Bone marrow—malignant cells

(*To face page 170*)

The cells occur in clumps are oval or polyhedral with basophilic cytoplasm Typical plasma cells may be seen and other cells which probably belong to the same series It is not necessary to actually penetrate into a myeloma when performing marrow puncture since the excess of lymphocytes and nests of typical myeloma cells make the diagnosis certain The blood usually shows a desaturated microcytic anaemia with a few normoblasts present There may be a slight leucocytosis due to lymphocytes and a few plasma cells

A characteristic of myelomatosis is the production of abnormal globulins, which in the case of cryoglobulin may crystallize out in the cold *appearing as needle like crystals inside the myeloma cells* Cryoglobulin also occurs in kala azar trypanosomiasis and other conditions The increase in globulin shown by a reversed A/G ratio causes clumping of red cells in the counting chamber when Hayem s fluid is used as a diluent

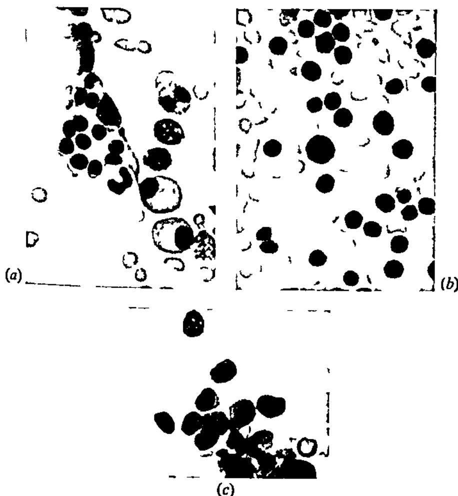
Macroglobulinaemia

A condition of middle and old age which may at first be mistaken for aleukaemic leukaemia is termed macroglobulinaemia since it is characterized by the production of large amounts of abnormal globulin There is a slight anaemia complicated by an haemorrhagic tendency and there may be a slight lymphocytosis in the peripheral blood The marrow shows an excess of lymphocytic cells which may be fragile so that they appear cracked A few plasma cells may also be seen in association with the clumps of lymphocytes The presence of euglobulins in large amounts can be demonstrated by the formol gel test or more simply by dropping the viscid serum into water Normally only a slight haze develops but with macroglobulinaemia a thick precipitate occurs which rapidly sinks

Megakaryocytic leukaemia

Fragments of megakaryocytes may appear in the peripheral blood in many of the leukaemias but it would not appear that a true leukaemia of platelet forming tissue exists Such cases as are described fit very well with the disorder myelosclerosis in which there is an increase of fibrous tissue in the marrow causing a progressive anaemia The platelet count may rise to 2 000 000 per c mm and actual megakaryocyte fragments be found in peripheral blood Immature red and white cells are also found in the blood in numbers out of proportion to the degree of anaemia The marrow is hypo plastic but megakaryocytes are present in large numbers The spleen and liver become active centres of haemopoiesis in this condition

PLATE II



(*a*) Bone marrow—myelomatosis (*b*) Peripheral blood—lymphatic leukaemia
(*c*) Bone marrow—malignant cells

(*To face page 170*)

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(a) Bone marrow—myelomatosis (b) Peripheral blood—lymphatic leukaemia
(c) Bone marrow—malignant cells

(*To face page 170*)

Leukaemoid reactions

Some conditions although not leukaemia produce a blood picture which strongly resembles one of the types of leukaemia the main one being myeloid This leukaemoid reaction is seen in myelo sclerosis (*see* above) and in other conditions Whooping cough often produces a blood picture resembling chronic lymphatic leukaemia and other diseases of children may do the same Acute sepsis tuberculosis and some poisons produce a myeloid leukaemia blood picture Probably the commonest leukaemoid blood picture is the leuco erythroblastic anaemia of metastatic deposits in the bone marrow There is anaemia with signs of blood regeneration normoblasts and myelocytes in the peripheral blood The platelet count is reduced and the marrow shows a normoblastic reaction with early haemoglobinization Clumps of malignant cells may be seen in the marrow but it is not possible to be dogmatic about the primary growth from their morphological characteristics

THE STORAGE RETICULOSES

A group of familial diseases characterized by hepato splenomegaly found most commonly in Jews are termed storage reticuloses since an inborn error of metabolism causes lipoid material to be stored in the reticuloendothelial system

Gaucher s disease

A mild hypochromic anaemia leucopenia and thrombocytonaemia associated with the presence of lipoid material (kerasin) in the cells of spleen liver lymph nodes and in any part of the reticuloendothelial system characterizes Gaucher s disease The typical Gaucher cell seen in marrow or spleen puncture is 20–80μ in size with a network of fine fibrils running lengthwise in the cyto plasm The deeply staining nuclei single or multiple are often found at the edge of the cell

Niemann Pick disease

In this condition also there is a mild hypochromic anaemia but associated with a leucocytosis Some cells in the peripheral blood may show lipoid material Bone marrow puncture reveals myeloid hyperplasia and masses of yellowish foam cells 30–60μ in diameter The nucleus is reticular and the foamy structure of the cytoplasm due to fine droplets of phosphatide material

Hand Schuller Christian disease

This condition otherwise known as essential xanthomatosis is characterized by bony defects exophthalmos and diabetes insipidus Anaemia is rare but there may be leucopenia and thrombocytopenia Sternal or spleen puncture reveals the characteristic cell very similar to that of Niemann Pick disease but containing cholesterol esters The cells may be found by puncture of the bony lesions

Infectious mononucleosis and glandular fever

The terms infectious mononucleosis and glandular fever have been used synonymously for many years It would appear, however that there are differences between the glandular fever described by Pfeiffer as an infectious disease of children characterized by generalized lymphadenopathy and the infectious mononucleosis of Sprunt and Evans a disease of young adults of low infectivity and lymphadenopathy Glandular fever produces a lymphocytosis with few abnormal cells and often no heterophile antibody Liver damage does not usually occur in this condition Infectious mono nucleosis on the other hand produces a lymphocytosis with more than 10 per cent of abnormal cells heterophile antibody is present in the serum in most cases and liver damage almost invariable The abnormal cell resembles a primitive monocyte often with an oval or horseshoe shaped nucleus with bluish grey abundant cytoplasm with a fenestrated appearance The edges of the cell are irregular and deeper staining than the rest of the cytoplasm The heterophile anti body is an agglutinin for sheep cells

In the invasive stage of the disorders there is a neutrophil leuco cytosis of 20–30 000 per c mm but the cells are the stab cells of Schilling and disappear to be replaced with lymphocytes The general picture has only one recognizable characteristic apart from the mononuclear cells and that is pleomorphism of the lymphocytic nuclei As a complication thrombocytopenia has been reported and haemolytic episodes have also been recorded

Paul Bunnell test —The presence of heterophile antibodies in the serum of sufferers from infectious mononucleosis is an important diagnostic feature Heterophile antibodies react with antigens totally unrelated to those which called them into being Agglutinins for sheep cells are heterophile agglutinins and may be present to low titre normally in human serum or can be produced by sensitization with horse serum or by the causative agent of infectious mono nucleosis Differentiation between the three antibodies is possible because the antibody of serum sickness is a Forssman antibody, the

antigen for which is widely distributed in nature Living things are divided into the guinea pig group which possess the antigen and the rabbit group which do not Treatment of a suspect serum with guinea pig or horse kidney suspension results in the antibody being absorbed if it is of Forssman type If ox cells are used for the absorption both Forssman antibodies and those of infectious mononucleosis are removed The patient s serum must be inactivated before the test to ensure destruction of complement

Infectious lymphocytosis

An acute and chronic form of lymphocytosis has been described which may have a total white count of 100 000 per c mm It occurs in children and rarely in adults and although appearing in blood and even marrow to be leukaemic the blood picture and marrow revert to normal with complete recovery The Paul Bunnell test is negative in these cases

DIFFERENTIATION OF ABNORMAL LEUCOCYTES

Alkaline phosphatase activity may be used to differentiate leukaemoid and infective states from the leukaemias Alkaline phosphatase activity is confined to the more mature granulocytes in normal blood and is greatly diminished in chronic myeloid leukaemia In infective states leukaemoid reactions and myelosclerosis the alkaline phosphatase level is very high The demonstration of the enzyme depends on the formation of an insoluble coloured precipitate where hydrolysis of a suitable substrate has occurred

Culture of blood and marrow is a certain method of identifying blast cells which are morphologically indistinguishable A simple culture medium is used and the behaviour of the cells observed by phase contrast the development of myelocytes from blast cells being sufficient to label them myeloblasts The technique is also used to differentiate normoblast and megaloblast

TECHNIQUE

LEUCOCYTE AGGLUTININS

Method

The method for demonstrating leucocyte agglutinates is as follows

(1) Defibrinate normal blood which is ABO compatible with the patient
(2) Add one fifth volume of 6 per cent Dextran solution (M W 150 000) and allow to sediment 30 minutes at 37 C in a tube tilted at 45 degrees

(3) Draw off the supernatant serum which should contain 6–10 000 white cells per c mm
Should the count be low the cells may be concentrated by centrifuging at 1 000 r p m for 3 minutes and discarding some of the serum before resuspending the cells
(4) To 0 1 ml of serum inactivated at 56 C for 20 minutes add 0 05 ml of leucocyte suspension Repeat with normal serum
(5) Incubate both test and control 1 hour at 37 C
(6) Add 0 1 ml leucocyte diluting fluid and examine for agglutinates in counting chamber

PAUL BUNNELL TEST

Details of the Paul Bunnell test using three reagents are given below

(*a*) *Guinea pig kidney emulsion*

(1) Remove capsules and fat from at least two pairs of kidneys and wash well with running water
(2) Place the kidneys in a small sieve and force through by rubbing with a pestle into a small quantity of saline
(3) Autoclave at 15 lb for 20 minutes and re sieve to obtain a fine suspension
(4) Wash the kidney suspension twice in saline and resuspend the deposit in four times its volume of 0 5 per cent phenol in saline

(*b*) *Ox red cell suspension*

(1) Defibrinate ox blood and wash the cells in several changes of saline
(2) Prepare a 30 per cent suspension of cells in saline and autoclave at 15 lb for 20 minutes
(3) Strain the cooled suspension through muslin and on the packed cell volume adjust to 20 per cent with saline
(4) Add an equal volume of 1 0 per cent phenol saline to give a final 10 per cent suspension in 0 5 per cent phenol saline

(*c*) *Sheep cell suspension*

(1) Wash sheep red cells three times with saline
(2) Adjust to 0 4 per cent suspension in saline The sheep blood should be less than 7 days old

Method

(1) Dilute guinea pig kidney suspension on packed cell volume so as to obtain a final 1 6 suspension in phenol saline
(2) Dilute ox-cell suspension to 4 per cent in phenol saline
(3) Inactivate patient s serum at 56 C for 20 minutes
(4) Place 0 25 ml amounts of inactivated serum in three tubes

(5) To the first tube add 1 0 ml saline to the second 0 75 ml saline and 0 25 ml guinea pig kidney suspension and to the third 1 0 ml of ox-cell suspension
(6) Mix the contents of the tubes and place in refrigerator at 4° C for at least 2 hours
(7) Centrifuge and retain the supernatants which constitute absorbed serum diluted 1 5 and a saline dilution of 1 5

Test proper

(1) Make serial dilutions of the three supernatants in saline to 9 tubes
(2) To each tube add one volume of 0 4 per cent sheep cell suspension and mix
(3) Incubate 2 hours at 37 C and read results macroscopically by gently flicking the tube with the finger and examining the agglutin ates in the concave microscope mirror

N B Glandular fever antibody is not absorbed with kidney but is partially absorbed with ox cells

Screening test

(1) Absorb inactivated serum with guinea pig kidney for 1 hour at room temperature
(2) Centrifuge remove the supernatant and to it add an equal volume of 0 4 per cent sheep cell suspension
(3) Leave the test at room temperature for 15 minutes then centrifuge for 2 minutes at 1 000 r p m
(4) Gently tap the tube and examine with a concave mirror The test is regarded as negative if no agglutination is seen but should agglutination be present at this stage a full absorbed technique is used

N B Known positive sera should always control the reagents in the Paul Bunnell test

LEUCOCYTE ALKALINE PHOSPHATASE (HAYHOE AND QUAGLINO)

The following are details of the leucocyte alkaline phosphatase test using the three reagents shown

(*a*) *Substrate*

Sodium *a* naphthyl phosphate	35 mg*
Brentamine Fast Garnet	35 mg
Working 0 05 M propanediol buffer	35 ml

(*b*) 0 2 *M propanediol buffer*

2 amino 2 methyl propane (1 3)-diol	10 5 g
Distilled water	500 ml

(3) Draw off the supernatant serum which should contain 6–10 000 white cells per c mm
Should the count be low the cells may be concentrated by centrifuging at 1 000 r p m for 3 minutes and discarding some of the serum before resuspending the cells
(4) To 0 1 ml of serum inactivated at 56 C for 20 minutes add 0 05 ml of leucocyte suspension Repeat with normal serum
(5) Incubate both test and control 1 hour at 37 C
(6) Add 0 1 ml leucocyte diluting fluid and examine for agglutinates in counting chamber

PAUL BUNNELL TEST

Details of the Paul Bunnell test using three reagents are given below

(a) *Guinea pig kidney emulsion*

(1) Remove capsules and fat from at least two pairs of kidneys and wash well with running water
(2) Place the kidneys in a small sieve and force through by rubbing with a pestle into a small quantity of saline
(3) Autoclave at 15 lb for 20 minutes and re sieve to obtain a fine suspension
(4) Wash the kidney suspension twice in saline and resuspend the deposit in four times its volume of 0 5 per cent phenol in saline

(b) *Ox red cell suspension*

(1) Defibrinate ox blood and wash the cells in several changes of saline
(2) Prepare a 30 per cent suspension of cells in saline and autoclave at 15 lb for 20 minutes
(3) Strain the cooled suspension through muslin and on the packed cell volume adjust to 20 per cent with saline
(4) Add an equal volume of 1 0 per cent phenol saline to give a final 10 per cent suspension in 0 5 per cent phenol saline

(c) *Sheep cell suspension*

(1) Wash sheep red cells three times with saline
(2) Adjust to 0 4 per cent suspension in saline The sheep blood should be less than 7 days old

Method

(1) Dilute guinea pig kidney suspension on packed cell volume so as to obtain a final 1 6 suspension in phenol saline
(2) Dilute ox cell suspension to 4 per cent in phenol saline
(3) Inactivate patient s serum at 56 C for 20 minutes
(4) Place 0 25 ml amounts of inactivated serum in three tubes

Sol 2	Potassium dihydrogen phosphate (KH_2PO_4)	0 6 g
	Disodium hydrogen phosphate (Na_2HPO_4 $2H_2O$)	0 6 g
	Dextrose	10 0 g
	Stock phenol red solution	100 ml

Make up to 400 ml with demineralized water Add the salts in the order shown and when dissolved add to Sol 1

Sol 3	Calcium chloride anhydrous ($CaCl_2$)	1 4 g
	Demineralized water	200 ml

Dissolve the salt and add to Sol 1 then filter through Seitz E K sterilizing disc and tube aseptically in 10 ml amounts

(*b*) *Stock phenol red solution*

Phenol red	0 2 g
N/10 sodium hydroxide (NaOH)	2 82 ml
Demineralized water to	100 ml

Mix the dye and alkali in a mortar and grind Add the water and filter through Whatman No 1 paper

(*c*) *Bicarbonate solution*

Sodium bicarbonate ($NaHCO_3$)	1 4 g
Demineralized water	100 ml

Distribute in 2 5 ml amounts and autoclave at 10 lb for 10 minutes

(*d*) *Hank s solution—normal strength*

Hank s solution 10 N	10 ml
Sterile demineralized water	90 ml

Mix and discard 2 5 ml then add 2 5 ml sterile bicarbonate solution Then add penicillin and streptomycin to a final 100 units per c mm

(*e*) *Culture medium*

Hank s solution normal strength	90 ml
Patient s sterile serum	10 ml
Bacto yeast extract	0 1 g

Method

Collection of material—Puncture marrow and expel aspirate into approximately 1 0 ml of patient s own serum containing 1 drop of heparin (5 000 1 μ/ml)

Slide preparation—The slides are machined from Perspex and consist of 5 mm thick Perspex The centre of the slide is drilled to provide a cavity 3 5 mm deep and a central pillar of Perspex 3 mm in height is sealed in the cavity The pillar is lipped the edge being 0 75 mm in height Two holes from the edge of the slide are drilled to the base of the cavity

(c) *Working buffer*

0 2 M solution (b)	25 ml
0 1 N hydrochloric acid	5 0 ml
Distilled water	100 ml

Method

(1) Fix fresh air dried blood or marrow films for 30 seconds in 10 per cent formalin in absolute methanol at 0 ± 5° C
(2) Prepare substrate and within 3 minutes filter directly on to the slides
(3) Incubate 5–10 minutes in substrate at room temperature
(4) Rinse the slides with tap water for 10 seconds
(5) Counterstain the dry slides for 15 minutes with methyl green
(6) Wash with tap water dry and mount in glycerol
(7) Counterstain Methyl green 2 per cent solution in distilled water The stain must be free of methyl violet

GLAND BIOPSY

(1) Cut the fresh gland across and place a cut surface on a slide and press gently
(2) Remove the tissue and dry the impression smear in air
(3) Stain by any of the approved methods

BUFFY COAT PREPARATIONS

(1) Collect defibrinated blood and centrifuge in a tube ½ in in diameter at 3 000 r p m for 15 minutes
(2) Remove the top layers of sedimented cells and pipette into haema tocrit tubes
(3) Centrifuge at 3 000 r p m for 30 minutes
(4) Make smears from the buffy coat and stain by any of the approved methods

FORMOL GEL TEST

(1) Place a small drop of patient s serum in the centre of a slide
(2) Invert the drop of serum over the mouth of a formalin bottle
(3) After 2 minutes examine the serum for evidence of gellification

SLIDE CELL CULTURE OF BONE MARROW

Slide cell cultures of bone marrow are obtained by the following methods using the five named reagents

(a) *Hank s balanced salt solution* (10 *N*)

Sol 1	Sodium chloride (NaCl)	80 g
	Potassium chloride (KCl)	4 g
	Magnesium sulphate ($MgSO_4$ $7H_2O$)	2 g

Dissolve in 400 ml demineralized water

CHAPTER 11

THE INVESTIGATION OF RHEUMATISM AND ALLIED CONDITIONS

TESTS

INTRODUCTION

It is convenient to include the investigation of disseminated lupus erythematosus under this heading although the condition is regarded as an allergic reaction and is not related to rheumatism Tests described in this Chapter are the erythrocyte sedimentation rate, the Rose Waaler test and the demonstration of L E cells

ERYTHROCYTE SEDIMENTATION RATE

Blood collected into anticoagulant and allowed to stand undisturbed shows the phenomenon of sedimentation of the red cells the rate of fall depending on many variables Primarily sedimentation is caused by rouleaux formation the weight of the cell aggregates causing them to sink at increasing speed until a constant rate of fall is reached This is maintained for a short while then packing of the sedimented cells begins and the rate slows until it finally ceases

The sedimentation rate is subject to a sex variation, increasing during pregnancy and often during menstruation It is increased in any case of tissue destruction in acute and chronic infections carcinomatosis operations, fractures irradiation and after injections of vaccines and foreign proteins The rate is also increased in rheumatic conditions but not in osteoarthritis It is slowed in polycythaemia and congestive heart failure increased blood viscosity probably playing a part

Value of test

The test is of value in the differentiation of organic disease from the symptoms produced in neurotic conditions and prognosis in the treatment of tuberculosis and rheumatism In the latter case successful treatment is indicated by a reduction in the sedimentation rate but it is essential that the same technique be used throughout treatment since results by different techniques are not comparable

(1) Melt 3 0 per cent New Zealand agar in demineralized water and add 0 3 ml agar to 0 9 ml of warmed culture medium Mix thoroughly and add a drop to the central pillar of the slide
(2) Cover with a cover slip so that the agar sets and produces a flat upper surface at the correct level Remove the cover slip carefully disturbing the agar as little as possible
(3) Pour the marrow into a watch glass select suitable fragments and transfer to the surface of the agar with the aid of a Pasteur pipette
(4) Replace the cover slip and seal with white beeswax
(5) The side holes may be used to introduce fluid medium either im mediately or at some future date in which case they are also sealed with beeswax
(6) Incubate the slides at 37 C or place on a roller device at 37 C Observation is made by phase contrast of a warm stage

minutes The normal sedimentin index is 0 5 or less Della Vida makes use of Day s sedimentin index to correct for anaemia by arithmetic proportion taking 45 per cent as the normal packed cell volume

$$\text{Sedimentin index} \doteq \log (\text{S R} \times 100)\,\frac{100 - 45}{100 - \text{P C V}}$$

$$\text{Corrected E S R} = \frac{\text{antilog S I}}{100}$$

The techniques principally used in this country are those of Wintrobe and Westergren The Wintrobe method uses Heller and Paul s mixture as an anticoagulant and has the advantage that the packed-cell volume can be determined on the same sample

Correction for anaemia is made from the chart of Whitby and Hynes based on dilution of normal blood Westergren uses sodium citrate solution as an anticoagulant and a higher column, this having value in high sedimentation rates

Since anaemias vary so much in relation to their sedimentation rates correction by dilution charts would not seem to be of consistent value It is suggested that using Wintrobe s method the test be reported normally plus the packed cell volume and the corrected rate The clinician on his own knowledge of the type of anaemia can then decide if the correction is valid

THE ROSE WAALER TEST

Rheumatoid arthritis sera are capable of agglutinating to high titre sheep red cells which have been sensitized with a sub agglutinating dose of homologous antiserum prepared in the rabbit The sensitized cells are added to the serum dilutions and results read on the pattern of agglutinates The patient s serum must be inactivated at 56° C for 5 minutes only since overtreatment leads to negative results

Most cases of rheumatoid arthritis give positive results, early ones often being negative Positive results are sometimes found in disseminated lupus erythematosus, scleroderma and pulmonary fibrosis

The Rose Waaler test can be modified by setting up a control titration with unsensitized cells the result being expressed as the ratio between the two titres This is the differential agglutination titre or DAT (Greenburg) Another modification uses sheep serum as a diluent instead of saline a process which enhances the titre of positive sera and may even produce a positive result where the saline titration is negative *Sheep sera vary in their suitability the best*

Factors influencing sedimentation rate

The sedimentation rate is influenced by the anticoagulant citrate solutions causing retardation when compared to solid anticoagulants and dry potassium oxalate causing red cell shrinkage thus making correction for anaemia difficult by alteration of the packed cell volume Heparin Heller and Paul s double oxalate mixture and EDTA used in the same technique give comparable results The red cell shape and size alter the rate considerably cells failing to rouleaux by reason of their shape (sickle cells) causing retardation and marked anisocytosis producing much the same effect A hypothetical plasma factor termed sedimentin has been postulated which is said to govern the size of rouleaux and hence rate of fall This substance loses its effect as the cells age so that clumping does not take place in blood over a few hours old with retardation of the sedimentation rate The effect is much less marked if Sequestrene is used as an anticoagulant It is also assumed that the greater the red cell anaemia the more sedimentin is available per cell but all anaemias do not behave as if this were true Temperature affects the rate of fall considerably and to ensure comparable results over a period of climatic change the tests should be performed in an incubator at 18–20 C Certain considerations affecting the apparatus must also be mentioned The tubes must have an internal bore of more than 2 5 mm to obviate capillary action they must be kept vertical With high sedimentation rates the column of blood should be as high as possible otherwise packing begins before a constant rate of fall can be determined

Methods

Many techniques have been elaborated for what must be regarded as a test having limited value The techniques vary in respect of the anticoagulant the height of the column and the method of reporting the results from one method not being comparable with those of another

Linzenmeier uses a column of 50 mm and sodium citrate solution as the anticoagulant reporting the time taken for the cells to fall to the 18 mm mark Cutler also uses a column of 50 mm and sodium citrate but measures the sedimentation rate at 5 minute intervals and plots the readings on a graph The method of reporting is interpreted in terms of activity of a tuberculous lesion the steeper the curve the more active the disease Day also plots readings at intervals and calculates a sedimentin index which is defined as the logarithm of the maximum rate of fall expressed in mm per 100

minutes The normal sedimentin index is 0 5 or less Della Vida makes use of Day s sedimentin index to correct for anaemia by arithmetic proportion taking 45 per cent as the normal packed cell volume

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having the property of producing a 2–8 fold enhancement in titre of test sera but no enhancement of agglutination by the sensitizing rabbit serum

DEMONSTRATION OF L E CELLS

Lupus erythematosus cells (Frontispiece) are neutrophils in the cytoplasm of which is an ingested spherical mass of nuclear material which has pushed aside the neutrophil nucleus The ingested material shows no structure and appears as a smooth homogenous mass staining purple with Romanowsky stains Sufferers from the disease possess a plasma factor which on incubation with cells releases desoxyribonuclease from combination with an inhibitor This enzyme is normally present in leucocytes and is capable of causing depolymerization of the desoxyribonucleic acid of neutrophils The nuclei thus affected are ingested by other neutrophils thus producing L E cells Preparations may be made either from incubated clotted or defibrinated blood anticoagulants seeming to be inhibitory The cells cannot be demonstrated in fresh blood or marrow and are only found in incubated blood or marrow of disseminated lupus erythematosus but the Tart cell may be found in health This is usually a monocyte which has phagocytosed another nucleus but without lysis The ingested material is either definitely lymphocytic with no evidence of lysis or is small dense and pyknotic Phagocytes may sometimes be seen surrounding masses of nuclear debris and are then termed rosettes

TECHNIQUE

ERYTHROCYTE SEDIMENTATION RATE

Methods of determining the erythrocyte sedimentation rate are as follow

Wintrobe method

(1) Collect venous blood into Heller and Paul double oxalate mixture
(2) Mix thoroughly and fill Wintrobe haematocrit to 100 mm mark
(3) Set the haematocrit vertically in a rack at 18–20 C
(4) At the end of 1 hour read the height of clear plasma in mm

Normal levels by this method
Males 0–9 mm in 1 hour Females 0–20 mm in 1 hour

Westergren method

(1) Collect venous blood into one fifth of its volume of 3 8 per cent sodium citrate

(2) Fill the Westergren tube to the 200 mm mark
(3) Set the tube vertically in a rack at 18–20° C
(4) Read the height of the clear plasma at 60 minutes and again at 120 minutes

Normal levels by this method
Males 3–5 mm in 1 hour 7–15 mm in 2 hours Females 4–7 mm in 1 hour 12–17 mm in 2 hours

The Westergren tube is a pipette 30 cm in length calibrated from 0 to 200 mm It is filled by suction and the finger held to the top until the end of the pipette is firmly resting on a rubber foot in the rack The top of the tube is secured with a spring clip

Micro sedimentation method

Reagent

Sodium citrate 5 per cent aqueous solution

Apparatus Micro sedimentation apparatus (Hawksley & Sons Ltd)

(a) Stand
(b) Celluloid plate 1 inch square
(c) Pipettes of 2 5 mm bore marked C and B and graduated 0 to 25 mm To mark C the pipette contains 0 04 ml and from C to B 0 16 ml

Method

(1) Fill pipette to mark C with sodium citrate solution
(2) Allow blood from a freely flowing ear prick to flow into pipette until it reaches the B mark
(3) Discharge contents of pipette on to celluloid plate and mix thoroughly with the pipette point
(4) Let mixture flow up pipette to the 0 mark by holding pipette horizontally
(5) Close flat end of pipette with the finger and place in rack
(6) Take reading at end of 1 hour

Normal by this method 6–8 mm for children and adults 8–10 mm for infants

ROSE WAALER TEST

Details of the Rose Waaler test are as follow

Reagents

(a) Sheep cells washed three times with normal physiological saline
(b) Rabbit anti sheep red cell serum of high titre prepared by giving intravenous injections of 5 per cent sheep cells at 5 day intervals and bleeding a week later Commercial preparations are available
(c) Patient s inactivated serum (3–10 minutes at 56 C)
(d) Small round bottomed tubes or Perspex agglutination trays
(e) Normal physiological saline

having the property of producing a 2–8 fold enhancement in titre of test sera but no enhancement of agglutination by the sensitizing rabbit serum

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Westergren method

(1) Collect venous blood into one fifth of its volume of 3 8 per cent sodium citrate

DEMONSTRATION OF LUPUS ERYTHEMATOSUS CELLS

L E cells are demonstrated by the following method

(1) Collect defibrinated blood by taking 10 ml venous blood into a flask equipped with a wooden applicator to which are attached several paper clips Rotate flask continually until fibrin clot is attached to paper clips
(2) Remove clot attached to paper clips and incubate the red cells in plasma at 37° C for 2 hours
(3) Centrifuge at 3 000 r p m for 10 minutes in tubes measuring 4 inches by ½ inch
(4) Using a capillary pipette remove the buffy coat together with some plasma and red cells and transfer to two haematocrit tubes
(5) Centrifuge at 3 000 r p m for 20 minutes
(6) Make films from the buffy coat plus a little plasma and stain by a Romanowsky method

Note Examination of several preparations is necessary before the test is regarded as negative It is recommended that negative cases should be repeated a further two times since experience has shown that L E. cells may not be present at all stages of the disease

Titration of sensitizing serum (rabbit anti sheep red cell serum)

(1) Make two sets of doubling dilutions one starting at 1 50 and the other at 1 75 This is the easiest way to prepare a series running from 1 50 1 75 1 100 1 150 1 200 etcetera to 1 2 000
(2) To each dilution add an equal volume of 2 per cent washed sheep cells and mix
(3) Incubate at 37° C for 1 hour
(4) Read by pattern of agglutinates on the bottom of the tubes

The first tube in which the cells have sedimented in a neat button of cells (negative reaction) indicates the dilutions at which serum should be used

Sensitization of sheep cells

Mix equal volumes of 2 per cent sheep cells and rabbit serum at the dilution obtained by foregoing technique Sensitization takes place within a few minutes

Test proper

(1) Make two sets of double dilutions of the inactivated serum
(2) To each tube of one set add one volume of sensitized cells and to each tube of the second set add one volume of unsensitized cells as a control
(3) Incubate at 37 C for 1 hour and then place in refrigerator at 4 C until cells have sedimented
(4) Read by pattern of agglutinates

The end point is taken at 50 per cent agglutination and is a pattern intermediate between the completely agglutinated and completely negative appearances The initial dilution of the test serum showing this appearance is reported as the titre of the serum Should the titre of the test containing unsensitized cells be eight or more the test serum should be absorbed with an equal volume of washed packed sheep cells and the test repeated

Result—Serum titres of 16 or more are regarded as positive

Note Known positive controls should be set up with each batch of tests

Differential agglutination titre

The te is performed as before and ratio calculated from end points for example first row sensitized cells 1 2 048 second row unsensitized cells 1 16 D A T = 1 128 (16 2 048)

Result—Differential agglutination titre of 1 16 or more is regarded as positive 1 8 is a borderline result and should be repeated a week later 1 4 or less is negative but should be repeated later if clinical signs and symptoms warrant it

invades the red cells and monocytes assuming odd shapes and appearing to have pointed ends The disease is transmitted by sand flies and an acute and chronic form exist

FUNGI

Histoplasmosis is a *rare condition* caused by *infection* with *Histoplasma capsulatum* In the tissues the organism appears to be a yeast, the bodies having a saucer appearance and being phagocytosed by the large cells of the spleen, bone marrow and lymph glands It may be demonstrated in the peripheral blood in the cytoplasm of monocytes and neutrophils In cultures at 37° C the organism grows as a yeast but at room temperature produces mycelia with aerial hyphae The disease manifests itself by enlarge ment of lymph glands, liver and spleen anaemia and leucopenia

PROTOZOA

The protozoan infections of man include those due to plasmodia, flagellata and leishmania causing malaria sleeping sickness and kala azar and Oriental sore

Plasmodium

Four species of plasmodia have been found in man and are differentiated by the type of pyrexia produced and on morphological grounds *Plasmodium vivax* causes benign tertian (B T) malaria and *P falciparum* causes malignant tertian (M T) malaria Quartan malaria is caused by *P malariae* and ovale malaria by *P ovale*

A rise in temperature every third day gives the name tertian to the disease and is produced by infection with *P vivax P ovale* and *P falciparum* although the last named often gives rise to an irregular fever *P malariae* infection produces a fever every fourth day and is known as quartan malaria

The malaria parasite undergoes three cycles of development two of which occur in man The sexual cycle (sporogony) occurs in an anophelene mosquito and culminates in the formation of mature sporozoites which migrate to the salivary glands When it bites, the mosquito injects into the host saliva contaminated with these para sites which enter the general circulation and make their way to the liver in the cells of which the exo erythrocytic cycle begins In the liver cells the parasites grow developing numerous nuclei which become merozoites and are released into the blood stream The period of time during which the parasites are developing constitutes the incubation period of the disease and should not all the merozoites enter the blood stream at once the liver may remain the seat of the

CHAPTER 12

BACTERIAL AND PROTOZOAN INFECTIONS

INTRODUCTION

WHILE bacterial infections and their investigation are properly the province of another branch of laboratory technology certain organisms and protozoa by the very fact of their demonstration in blood and blood forming tissues must be regarded as within the realm of haematology The organisms thus classified include bacteria fungi, protozoa and the larvae of certain helminths

BACTERIA

The bacteria which can be demonstrated in the blood or blood forming tissues of infected man include types of spirochaetes and Bartonella the latter being related to the Rickettsiae

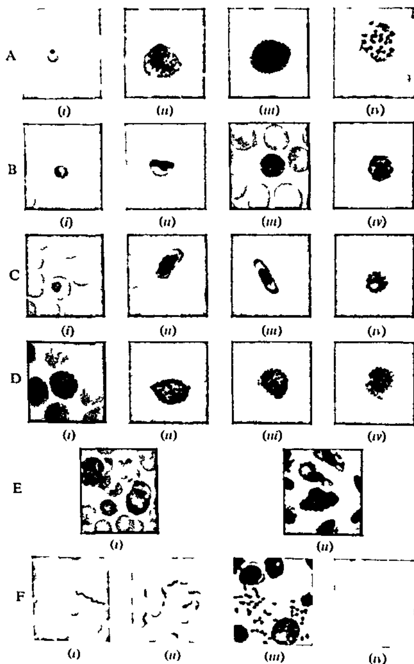
Borrelia recurrentis is the causative organism of European relapsing fever, a disease transmitted by lice The spirillum is 10–20μ in length with four or more spirals They may be seen bent in the middle in stained preparations and in fresh preparations sometimes turn in a circle The organism is demonstrated in the peripheral blood *Borrelia duttoni* is morphologically indistinguishable from *B recurrentis* and is the causative organism of African relapsing fever which is usually transmitted by ticks Blood films suspected of containing the parasites are best stained by a long Giemsa method

Leptospira icterohaemorrhagiae is the causative organism of Weil s disease and is carried by the water rat whose infected urine contaminates the water The organism is a very fine filament 5–25μ in length with very tight coils and the ends often curved giving the appearance of a walking stick Since the organism is only demonstrable in the blood early in the disease it is often missed Dark ground illumination is the most suitable method of demonstration

Spirillum minus the cause of rat bite fever of the Far East is a very small spirochaete 2–4μ in length with two or three curves The ends of the parasite tend to be pointed

Bartonella bacilliformis is the causative agent of Carrion s disease It is seen as small rods 1–2μ in length or coccal forms The organism

PLATE III



(*To face page 188*)

exo erythrocytic cycle with consequent relapses of the disease even after many years This is particularly the case with quartan malaria

On entering the blood stream the merozoites start the erythrocytic or asexual cycle which is sometimes termed schizogony The parasites attach themselves to and penetrate the red cells, appearing as small spherical bodies with a dot of chromatin This rapidly develops into the early trophozoite or signet ring form which grows into an amoeboid form or late trophozoite Pigment derived from the parasite and haemoglobin breakdown may appear at this stage The parasite then grows into a schizont in which the chromatin is shared among the cytoplasm forming merozoites which eventually cause rupture of the infected red cell and on liberation restart the erythrocytic cycle The gametocytes or sexual forms of the parasite also develop from the trophozoite but no fertilization takes place in the blood stream

The gametocytes are taken up by the mosquito during biting and exflagellation of the male parasite (microgametocyte) occurs in the vector s stomach The gametes produced by this process fertilize the female parasite (macrogametocyte) and an oökinete is formed capable of movement like a ciliate This form of the parasite penetrates *the stomach wall and forms an oocyst which when it ruptures* liberates numerous thread like sporozoites into the body cavity from where they make their way to the salivary glands

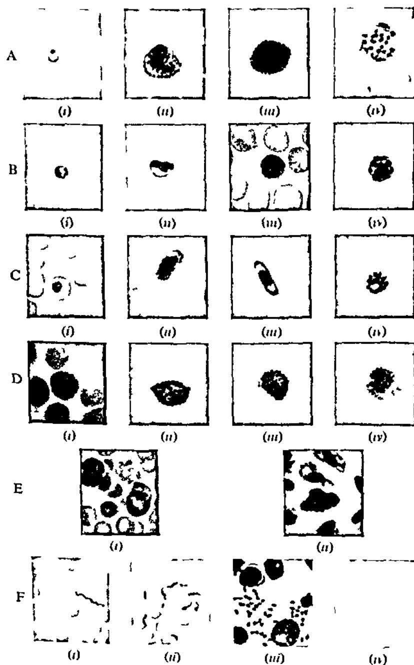
The types of malaria parasite are differentiated on morphological grounds In man the infected red cell is enlarged with *P vivax* and delicate red dots known as Schuffner dots appears early in the parasite Fine yellow brown pigment granules also appear in the amoeboid form of the trophozoite The schizont contains 18–24 merozoites and the large gametocytes are heavily pigmented In common with other varieties of the parasite the chromatin of the macrogametocyte is aggregated into one piece while that of the microgametocyte is dispersed throughout the cytoplasm All forms of *P vivax* are commonly seen in peripheral blood

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LEGEND TO PLATE III

A *Plasmodium vivax* (*i*) early trophozoite (*ii*) male gametocyte (*iii*) female gametocyte (*iv*) schizont B *Plasmodium malariae* (*i*) early trophozoite (*ii*) late trophozoite (band form) (*iii*) female gametocyte (*iv*) schizont C *Plasmodium falciparum* (*i*) early trophozoite (*ii*) male gametocyte (*iii*) female gametocyte (*iv*) schizont D *Plasmodium ovale* (*i*) late trophozoite (*ii*) male gametocyte (*iii*) female gametocyte (*iv*) schizont E (*i*) *Plasmodium muris* (*ii*) *Plasmodium gallinarum* F (*i*) *Borellia recurrentis* in blood (*ii*) *Trypanosoma cru i* in blood (*iii*) *Leishmania donovani* in spleen smear (*iv*) *Microfilaria bancrofti* in hydrocoele fluid

PLATE III



(To face page 188)

P ovale infection results in symptoms indistinguishable from infection with *P vivax* but the parasites are recognized by the fact that the infected red cell is not enlarged although it develops an oval shape with fringed ends Pigment granules develop early and the mature schizont contains few merozoites than *P vivax* All forms of *P ovale* are found in peripheral blood

P falciparum is characterized by lack of enlargement of infected red cells multiple infections of red cells with a finer ring form than other types Marginal forms of the early trophozoite may be seen appearing to adhere to the inner surface of the red cell Double dots of chromatin on the signet rings are common Red dots and streaks of material termed Maurer s dots or clefts may be seen in parasitized red cells Usually the early trophozoite is the only form of *P falciparum* seen in the peripheral blood but a search will usually reveal the characteristic crescent shape of the gametocytes The presence of the schizont containing 8–24 small merozoites in the blood stream is regarded as a bad sign, this form of the parasite usually being found in internal organs such as bone marrow spleen liver kidneys and brain capillaries

P malariae infection does not cause enlargement of the infected red cells and the band form of the late trophozoite is characteristic as is the schizont with its 8–12 merozoites arranged as a rosette The gametocytes closely resemble those of *P vivax* but completely fill the cell and are found in the marrow more often than in the peripheral blood

Blood picture

Leucopenia due to depression of the myeloid elements occurs and signs of blood regeneration are obvious in the stained film Since infection results in red cell destruction a severe attack produces the picture of a haemolytic anaemia The aetiology of blackwater fever is bound up with the administration of quinine to patients infected with *P falciparum* the disorder behaving as an acute haemolytic anaemia with passage of urine containing haemoglobin pigments

Diagnosis

A fever of regular rhythm shivering attacks and splenomegaly in a malarious area is almost sufficient for the diagnosis which is confirmed by the demonstration of the parasite in the blood Capillary blood taken while the temperature is rising shows the parasites best in some cases scarcity of the parasite makes thick films a necessity Owing to shrinkage in thick films the morphology of plasmodium differs from that seen in thin films Staining methods are discussed in Chapter 4

THE TRYPANOSOMES

Trypanosome infection causes African sleeping disease and Chagas disease of South America Sleeping sickness is transmitted by the bite of the tsetse fly and is caused by *Trypanosoma gambiense* or *Trypanosoma rhodesiense* Chagas disease caused by *Trypano soma cruzi* is transmitted by a bug of the genus Triatoma Natural *reservoirs of infection exist for the trypanosomes among African* game marsupials cats and dogs

It is necessary for a reproductive cycle to occur in the vector before it can transmit the disease to man which it does by biting A sore at the site known as the trypanosome chancre yields the parasite early in the disease and puncture of adjacent lymph glands also provides smears showing trypanosomes From the sixth to the twelfth day after infection the parasites may be found in the blood and later in the disease in the cerebrospinal fluid

Morphology

It is difficult in blood films to differentiate between *T gambiense* and *T rhodesiense* although far more parasites may be found in the latter infection The trypanosomes are fish like in shape 2–3 times as long as a red cell and contain a nucleus and a smaller structure called the blepharoplast from which extends a flagellum forming an undulating membrane Volutin granules are seen in the cytoplasm of the parasite Reproduction of *T gambiense* and *T rhodesiense* is by binary fission *T cruzi* is smaller than the other types and the blepharoplast much larger In the tissues it assumes a leishmania form (*see* below) and reproduces by mitotic division It may be difficult to demonstrate since the numbers in blood are small but they may be concentrated by a simple technique

The trypanosomes may be cultured and inoculated into laboratory animals In culture they assume a leptomonad form similar to the Leishmania

Thick or thin films of blood taken during the fever are best to demonstrate the parasites stained by any of the Romanowsky stains

THE LEISHMANIA

Leishmania are the causative agents of kala azar, oriental sore and cutaneous and mucosal leishmaniasis The vector is a sand fly of the phlebotomus family and cats and dogs act as reservoirs of infection Kala azar is a grave disease of tropical and sub tropical regions characterized by irregular fever and hepato splenomegaly Leucopenia and mononucleosis are indications of the condition

together with a severe anaemia The disease is fatal if untreated The variant of leishmania responsible is *Leishmania donovani*

Oriental sore is a localized form of cutaneous leishmaniasis characterized by the development from a papule of a painless ulcer, which heals after a prolonged period It is caused by infection with *Leishmania tropica* South American cutaneous and mucosal leishmaniasis is a mutilating disorder caused by *Leishmania braziliensis*

Morphology

The three variants cannot be distinguished from one another in films They appear in tissue as ovoid bodies about one third the size of red cells and are usually intracellular the host cell being a mono cyte or endothelial cell They contain a spherical nucleus and a rod like blepharoplast and are non motile In culture they assume a leptomonad form like trypanosomes with which they are related

Diagnosis

Kala azar may be diagnosed by the demonstration of the parasites in bone marrow or by spleen puncture They do not appear in the blood The ulcer of oriental sore and lesions of cutaneous leish maniasis may be punctured using a syringe and the fluid from the ulcer base aspirated This avoids contamination with pus and bacteria usual in these lesions The smears may be stained by any of the Romanowsky methods the cytoplasm appearing blue and the nucleus and blepharoplast reddish violet

The formol gel test (Chapter 10) is often positive in trypano somiasis and kala azar

HELMINTH INFESTATIONS

Many worm infestations produce haematological signs such as eosinophilia but only two can actually be demonstrated in the blood

Filaria

The mature helminths live in connective tissue or lymphatic channels and liberate embryos termed microfilaria into the blood stream The insect vector is a mosquito a reproductive cycle being necessary before the bite becomes infective to man

Wuchereria (*Filaria loa*)

The causative agent of Calabar swellings is the *Filaria loa* the adult form of which migrates in the tissues and has even been seen traversing the conjunctiva The parasite is 30 mm or more in length the female being longer than the male

Wuchereria bancrofti

This filaria, *Wuchereria bancrofti*, is the cause of elephantiasis due to obstruction of lymph channels by the adult worms The adult worm is 3–4 inches in length, the female being longer and thicker than the male After death the helminth becomes calcified in lymph glands and vessels

Acanthocheilonema perstans

This parasite, *Acanthocheilonema perstans* appears to exist with out symptoms in those infected It has been reported as causing subcutaneous cysts

Diagnosis

The condition of filariasis is diagnosed mainly on the presence of microfilaria in the blood stream *W bancrofti* embryos are seen in greater numbers during the night and may even not be seen at all during the day They seem to have a colourless sac or sheath around their length *Wuchereria loa* (*Filaria loa*) is very similar in size and shape and is also sheathed There is no nocturnal periodicity *Acanthocheilonema perstans* is a much smaller embryo and is not sheathed The parasites are demonstrated in wet preparations or stained with Giemsa A dotted staining down the length of the embryos is characteristic Filariasis always shows a high eosino philia

TRICHINELLA SPIRALIS

The infestation is contracted by eating measly pork in which the larvae lie encysted Mature worms appear in the intestine within 48 hours of ingesting the infected meat Embryos are rapidly pro duced and migrate via the lymphatics to the tissues A high eosino philia results and the embryos are occasionally demonstrated in blood They measure 100μ by 6μ and are thus easily differentiated from other embryos

TECHNIQUE

The following are details of test techniques relevant to bacterial and protozoan infections

CONCENTRATION OF MICROFILARIA

Reagent	5 per cent formalin	95 ml
	Glacial acetic acid	5 ml
	Sat alcoholic sol crystal violet	2 ml

Method

(1) Collect venous blood in 3 8 per cent sodium citrate
(2) To the blood add five times its volume of reagent
(3) Centrifuge and discard supernatant
(4) Examine deposit wet under a coverslip

CONCENTRATION OF TRYPANOSOMES

Reagent

Normal physiological saline containing 6 per cent sodium citrate

Method

(1) Collect 9 ml of venous blood into 1 ml of reagent as an anti coagulant
(2) Centrifuge at 1,500 r p m for 5 minutes
(3) Remove buffy coat with a capillary pipette and make films as for blood
(4) Stain by a Romanowsky method

STAINING OF SPIROCHAETES

(1) Prepare Giemsa staining solution of 1 part Giemsa to 19 parts of distilled water buffered to *p*H 7 2
(2) Fix films in methyl alcohol 5 minutes
(3) Pour on stain heated until it steams
(4) Replace with fresh hot stain after 15 minutes and leave 30 minutes
(5) Wash with distilled water drain and dry

CHAPTER 13

THE USE OF RADIOISOTOPES

THEIR APPLICATION IN HAEMATOLOGY

INTRODUCTION

IT IS NOT POSSIBLE to deal with the many applications of radioisotope techniques in a single chapter What follows is a condensed version of atomic theory a description of some of the apparatus used and a discussion of the use of radioisotopes particularly in haematology For fuller details the interested reader is referred to the excellent text books on the subject

ATOMIC STRUCTURE

The atom consists of a central nucleus surrounded by orbital electrons The chemical identity of the atom is determined by the number of electrons each one of which carries a unit negative electrical charge The nucleus consists of positively charged protons and uncharged neutrons Normally the atom possesses equal numbers of protons and electrons so that it is electrically neutral The atomic number of an element is given by the number of protons or orbital electrons but a given sample of the element may contain atoms carrying a greater or a lesser number of neutrons These variants are termed isotopes and are designated by their atomic weights for example O^{18}

RADIOISOTOPES

Chemically the isotopes of an element behave similarly but some of them known as radioisotopes are unstable They attain stability by gaining or losing a unit of electric charge and radiating away any excess energy The change of nuclear charge means that the element moves one place in the periodic table Such a nucleus is said to be radioactive and the form of the element a radioisotope It is possible to measure the radioactivity since the number of dis integrations per second is directly proportional to the number of radioactive atoms in the sample at that time The unit of radio activity is the Curie defined as $3\ 700 \times 10^{10}$ disintegrations per second the millicurie $3\ 7 \times 10^{7}$ and the microcurie $3\ 7 \times 10^{4}$

disintegrations per second The rate of decay of a radioisotope is usually described by the time taken for radioactivity to be reduced by one half This is termed the half life of the isotope and is important when planning experiments with these materials

RADIATION

The radioisotopes of present interest decay by emitting a β particle (fast electron) accompanied in some cases by γ or electro magnetic radiation All radiation is injurious especially to rapidly growing tissues such as the bone marrow and the sex cells Damage to haemopoietic tissue can be permanent and the effect on the gonads can only be assessed by mutations appearing in later generations The type of material used in clinical tracer work is not dangerous to the patient but the operator must avoid overexposure Stringent regulations govern the use and disposal of radioactive materials since problems of public health are involved Several publications dealing with radiation hazards are available from H M Stationery Office and the Radiological Protection Service will provide further information on request

PRODUCTION OF RADIOISOTOPES

Most tracer radioisotopes are produced by neutron irradiation in a nuclear reactor the process forming either an isotope of the same element or a different chemical element Other isotopes are prepared by bombardment of the target material with protons or α particles in a cyclotron Purification of the isotopes and synthesis of compounds to include the radioactive element in a desired position in the molecule constitutes a special branch of chemistry with its own problems

DETECTION AND MEASUREMENT OF RADIATION

Three methods of detecting and measuring radioactivity are available The Geiger Muller counter is primarily a β ray detector but γ radiation is also detected A circle of eight Geiger counters is termed a ring counter and is suitable for measuring radioactive samples in large bottles The scintillation counter is used for γ ray detection the property of producing a secondary electron in an irradiated absorbing medium being utilized The absorbing medium is a suitable optically transparent crystal and the excited electrons radiate their excess energy as light quanta suitable electronic equipment measuring the impulses The ionization chamber is the third method of detecting radiation and measures the small current produced when γ rays produce fast moving electrons which ionize the gas in the chamber

Complicated electronic equipment is necessary to record the impulses detected by the counters Background radiation due to cosmic rays or radiation from building materials must be noted and shielding fixed where necessary and due allowance must be made for background counts in all tests It is desirable for standard preparations to be measured at the same time as the test material The physics involved in the construction and maintenance of the apparatus are outside the scope of this book

THERAPEUTIC APPLICATIONS

The radioisotope of phosphorus, P^{3}, is a pure β emitter with a half life of 14 3 days Since it is selectively taken up by rapidly metabolizing tissue, including haemopoietic tissue, it has been used to treat polycythaemia by irradiation *in situ* The cobalt isotope Co^{60}, with a half life of 5 3 years, is used in the treatment of malig nant conditions either by means of wire introduced into the tumour, or by external teletherapy

RESEARCH PROJECTS

The radioisotope is a valuable research tool used in biological and physiological research The radioactive material may be incorporated in physiological compounds and their breakdown followed in the body, thus adding enormously to our knowledge of these processes

DIAGNOSTIC PROCEDURES

Since iron enters into the composition of haemoglobin radio isotopes can be used to measure its absorption storage and excretion The isotope Fe^{59} is used in such studies because of its relatively long half life of 45 days Normal individuals absorb very little of a test dose of radioiron polycythaemic patients no more than a trace while iron-deficient states show very much greater absorption The absorbed iron stored in body organs is detected by means of a directional (lead shielded) Geiger or scintillation counter and that taken up by red cells measured by the activity of blood samples in a scintillation counter Radioiron excreted in the faeces is measured in a ring counter

The plasma volume plasma iron turnover and red cell utilization may be determined by incubating Fe^{59} in the form of ferric chloride with β globulin in the patient s own plasma The resultant iron bound protein is injected into the patient and several blood samples taken in the next few hours and subsequently daily for 2 weeks Radioactivity in the plasma samples is measured in a scintillation

counter against a standard of the material injected In iron deficiency anaemia the plasma iron turnover is either normal or greater than normal with complete utilization of the injected iron

Red-cell survival studies in the haemolytic anaemias are undertaken by tagging the red cells with a radioisotope The technique is also of value in assessing the red cell preserving properties of new anticoagulants The cells are labelled with Cr^{51} by exposing them to a solution of the isotope in the form of sodium chromate and then re injected into the subject from whom they were obtained The red cells take up a considerable amount of the radioisotope but some is eluted from the cells on re injection Due correction must be made for this loss The activity of a blood sample taken 20 minutes after injection and daily samples after that is measured in a scintillation counter the standard being a sample of the original chromium preparation Normally the blood radioactivity drops to less than one half in 28–36 days due to the physiological destruction of cells which have reached their allotted life span An abnormal rate of red cell destruction is shown by a reduction of this time The radioisotope P^{3} is also used for red-cell survival studies

Vitamin B_1 labelled by the incorporation of Co^{58} is used for the *diagnosis of megaloblastic anaemias Radioactive B_{12} is given orally* together with carbachol intramuscularly to stimulate intrinsic factor secretion, and all faeces collected until no longer radioactive The radioactivity is measured using the ring counter the normal amount excreted being two thirds of a test dose of 1 μg If the excreted B_{12} is larger in amount than this a further dose is given together with an intrinsic factor preparation Again the faeces are collected and radioactivity determined In Addisonian pernicious anaemia and gastrectomy syndrome less than two thirds of the test dose is excreted because failure to absorb B_1 is due to lack of intrinsic factor In other malabsorption syndromes the intrinsic factor is available

The excretion of B_{12} in the urine (Schilling test) is also used in the diagnosis of the megaloblastic anaemias if it is not possible to collect faeces Radioactive B_{12} and carbachol are given followed by non radioactive B_1 All the urine passed in 24 hours is pooled and activity measured with the ring counter The normal absorption of B_1 is up to 90 per cent of the test dose *If less than 10 per cent is excreted* radioactive B_{12} is given together with intrinsic factor and followed by non radioactive B_1 The urine is collected as before and radio activity measured In pernicious anaemia and gastrectomy syndrome less than 10 per cent of the test dose is excreted in the urine but in steatorrhoea and other loop syndromes more than 10 per

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cent is still excreted Plasma radioactivity may be measured 8 hours after the test dose using a liquid scintillation counter and liver radioactivity after 7 days using a shielded scintillation counter over the liver

CONCLUSION

The use of radioisotopes in haematological diagnosis requires the close co operation of haematologist and physicist, since the apparatus used is both unfamiliar and complicated The inclusion of the uses of radioisotopes in the laboratory technologist s curriculum is an indication of this harmonious partnership

SECTION II

BLOOD TRANSFUSION TECHNIQUE

CHAPTER 14

BLOOD GROUP ANTIGENS AND ANTIBODIES

GENERAL

CHEMISTRY

MUCH has been written about the chemistry of the blood group antigens and antibodies and for full details the interested reader is referred to the appropriate publications Recent work by Morgan and Watkins (1959) suggests that the blood group substances are *muco polysaccharides specificity being closely connected with certain component sugars* It is possible that basic materials are available which are enzymatically modified to produce specific blood group substances the enzyme processes being genetically determined The blood group antibodies are globulins behaving mainly as γ *globulins*

The antigens are located on the red cells at hypothetical sites termed receptors and by appropriate means other receptors may be uncovered or donated The enzyme methods (*see* Chapter 8) are extremely useful in this respect but ficin and papain have the *property of almost completely destroying some antigens Blood* group antigens may be present in a water soluble form in which case they are present in the body fluids or in an alcohol soluble form when although not found in body fluids they may still be demonstrated in extracts of the organs

ANTIGEN ANTIBODY REACTIONS

The blood group antibodies act by attaching themselves to the red-cell receptors An optimal amount of antibody is required smaller amounts resulting in sensitization of the cells while the visible effect is *delayed and weak Although antibodies react in* many different ways the end result to be of value must be visible therefore no matter what techniques are used in blood group serology the final process is one of agglutination or lysis

Agglutinins

Agglutinins act by deposition of antibody on the cells until actual clumping occurs It will be obvious from the foregoing con

(Chapter 15) is principally involved but possible antibodies in other systems have been described where no immunization can be shown to have occurred Usually the naturally occurring antibodies have a thermal optimum of 20° C but may react better at 4° C and sometimes at 37° C The antibodies are usually saline acting and not of high titre Except under pathological conditions an antibody cannot exist with its corresponding antigen in the same blood naturally occurring antibodies arising in the absence of the antigen It is not known why these antibodies occur some authorities holding that they are genetically determined while others believe that they are really produced in response to immunization the process differing from artificial immunization resulting in a modified antibody

IMMUNE ANTIBODIES

Immune antibodies may be produced by injection which includes transfusion or by pregnancy The response to the introduction of a foreign antigen varies greatly in individuals and depends upon whether or not the antigen has been encountered before If the antigen is new to the host there may be little or no production of antibody but the body defences are put on guard This is termed the primary response A second or repeated injections of the antigen, however, may result in rapid production of antibody Some disorders cause greater liability to antibody production lupus erythematosus being well known in this respect The so called anamnestic reaction is a response to an apparent unrelated antigen causing other antibodies to increase in titre Investigations usually show that such antigens have a definite relationship to those specific for the affected antibodies

The means by which pregnancy causes antibody production is still obscure It has been shown many times that foetal red cells can cross the placenta and should they possess an antigen donated from the father and foreign to the mother it is possible that they may be the immunizing agent It is also possible that soluble group substances or even fragments of embryonic tissue may also act in the same manner in heterospecific pregnancies

Immune antibodies may be saline acting or albumin antibodies Prozone phenomena due to mixtures are common and antibodies of very high titre may be produced by immunization The immune antibodies of those systems which possess naturally occurring antibodies differ in their thermal optimum and often develop haemolytic properties

siderations that the strength of the red cell suspension is of para mount importance in such reactions too strong a suspension result ing in absorption of the antibody with minimal agglutination Agglutination may be intense and easily visible to the naked eye or may consist of small clumps in a sea of free cells The latter appear ance although only microscopic has the same importance and significance as macroscopic agglutination A serum containing anti bodies which react speedily to form large tightly packed red cell clumps, is said to be avid but a serum of high titre is not necessarily of good avidity

Lysins

Lysins do not destroy the cells but cause liberation of haemo globin by multiple punctures of the cell envelope presumably at receptor sites Complement is necessary for such reactions again an optimal amount being essential The lysin sensitizes the red cells, which on becoming loaded with complement are damaged thus set ting free the haemoglobin Fresh serum must be used for investiga tions involving lysins or a source of complement such as fresh guinea pig serum provided

Precipitin reactions

Precipitin reactions have a limited use in blood group serology, their principal value being in medico legal work Using the appro priate anti species precipitin sera it is possible to determine the animal from which a blood stain is derived The antigenic material from the red cell stroma precipitates out of solution on combination with the antibody

Prozone phenomenon

It sometimes happens that a serum fails to give a visible reaction at low titre but does so on further dilution This effect is termed a prozone phenomenon and is due to the action of an accompanying albumin antibody acting faster than the saline antibody In effect the two antibodies are competitors for the same receptors but the saline antibody being present in higher titre is revealed by dilution of the serum

NATURALLY OCCURRING ANTIBODIES

Certain of the blood group systems are associated with the presence of naturally occurring antibodies The ABO system

technique using optimal incubation times for the antibodies con cerned Precipitin tubes 50 mm × 11 mm are used for this purpose, they may be capped by short flat bottom tubes to prevent evaporation and to keep dust free Using tube techniques with rare antisera often involves working with very small quantities of material, a contingency which introduces handling difficulties Considerable experience is needed to handle an ordinary glass capillary pipette using volumes of the order of 0 01 ml and less, but control may be attained by the use of a throttle luted into the stem, or a fitted sintered glass disc (Fig 39)

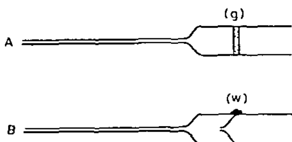


FIG 39—(A) Pipette fitted with sintered glass disc (g) (B) pipette with throttle (w) luted into stem with sealing wax

Microscopic agglutination

Care must be taken when reading microscopic agglutination In order that small clumps are not broken up and to ensure proper observation the sedimented cells should be laid gently in a rivulet across the width of a slide In this manner clumps of cells may be seen rolling free and their true nature determined The criterion of true red cell agglutination is that it occurs in three planes The stickiness which is sometimes seen involving small numbers of cells will be found on close inspection to be only in two planes Rouleaux formation occurs in individuals who have high sedimentation rates or who have received dextran and might cause confusion but the appearance of red cells looking like a pile of coins is typical and in many cases can be dispersed by gentle mixing with saline An interesting pseudo agglutination occurs in individuals with a reversed albumin/globulin ratio This appearance often seen in myelomatosis is not due to an agglutinin since the direct antiglobulin test is negative

Pan agglutination

Bacteriogenic pan agglutination is a phenomenon caused by the action of certain bacteria which uncover a latent receptor on the red cell This T receptor acts as an agglutinogen the corresponding

INHERITANCE

The blood group antigens are inherited mainly as simple Mendelian dominants Some closely linked genes are inherited as co dominants and in two blood group systems some doubt exists as to the mode of inheritance There is no sex linkage, and linkage between blood group genes is close so that crossing over can occur but is rare This accounts for the unexpected appearance of rare chromosomes There is some linkage with other inherited characteristics such as the ability to taste and smell certain compounds and this property makes the blood group genes useful as ethnological markers The inheritance of some blood group systems is on such firm ground as to make it useful in law and in fact is accepted in cases of disputed parentage in many parts of the world

Many interesting genetic phenomena are observed in the study of blood groups In addition to crossing over examples of chromosome deletion are found where a piece of chromosome carrying the gene or genes is lost resulting in a puzzling phenotype Such cases manifest stronger reactions with the remaining antigens of the group seeming to be able to utilize all the basic substance available for the complete group Chromosome deletions breed true, that is the loss of the chromosomal material when acquired becomes an inherited characteristic The activity of modifying genes causing partial or complete suppression of some blood group characteristics is accepted today and apparently inert phenotypes are sometimes explained by an allelomorph which is devoid of activity Such an allele is termed an amorph

TECHNICAL CONSIDERATIONS

Macroscopic agglutination may in some blood group systems occur so quickly and be so obvious that a simple tile technique will be adequate for its demonstration The glazed white tile is merely marked with grease pencil and rocked to mix the reagents Such preparations dry rapidly and cannot be incubated but the Murray slide, which consists merely of a heavy glass slide of alternate troughs and platforms is free from these objections The reagents *are applied to the platforms and rocked to mix* Breathing on the slide facilitates mixing and the slide is then incubated in a moist chamber easily contrived from a Perspex museum box containing a layer of damp lint The slide has the added advantage that it may be examined microscopically

Tile and slide techniques however should be confirmed by a tube

The scores for all tubes are summed and compared with the scores of known cells containing single and double doses of antigens Dosage tests also have other applications since they can be used to indicate whether or not a serum contains more than one antibody

Preparation of apparatus

Special cleaning of apparatus is necessary, since agglutinins are absorbed or inactivated by a variety of unrelated materials Washing-soda preparations are preferable to detergents which may produce lysis and inhibition of agglutination if not removed entirely from the glassware Tubes which are not sparkling clean when viewed in a good light must be rewashed It is recommended that used precipitin tubes be made up in bundles held by rubber bands and allowed to stay immersed in water overnight They are then placed in a bowl of a strong solution of Brylanz (G Gurr Ltd) ensuring that the tubes are filled, and boiled for 10 minutes After thorough rinsing in tap water they are then transferred to a weak solution (5–10 per cent) of hydrochloric acid for one hour After further thorough rinsing with tap water the tubes are rinsed in distilled water and dried mouth downwards in the hot air oven Blotting paper should cover the bottom of the crates to prevent the tubes falling out

REAGENTS

The reagents used in blood transfusion work are cells soluble antigenic material and sera Some of these are rare and yet must be on hand when needed for which reason they must be capable of storage *Soluble antigenic materials such as saliva are stored frozen* solid at $-20°$ C in small amounts as also are sera Under these conditions they retain their properties for a considerable time but red cells kept at $+4°$ C in dextrose saline lyse after a few weeks and lyse immediately on thawing from the frozen stage However by suspending the cells in a glycerine citrate mixture cells may be frozen solid without damage so that rare genotypes can always be available Dried sera are used occasionally having the advantages of mixing with cells without causing dilution and of being stored at $+4$ C

TECHNIQUE

The following are details of the techniques to which reference is made in this Chapter

agglutinin anti T being present in all adult human sera This is the Hubener Thomsen phenomenon and cells which have been modified by the action of the bacteria are agglutinated by all adult sera The antibody can be specifically removed from sera with the altered cells

Another type of bacteriogenic pan agglutination is caused by sera infected with various skin diphtheroids This causes the sera to develop an anti h agglutinin which reacts with an h antigen on all red cells This must not be confused with the anti H of the ABO system (*see* Chapter 15)

Estimate of agglutination strength

The strength of agglutination is best estimated by using a scoring system and that used at the M R C Blood Group Reference Laboratory is standard in Britain

Grades of agglutination

C (*complete*)	Clumps of cells in clear surrounding fluid visible to naked eye
V (*visual*)	Rather weaker clumping directly visible but with surrounding fluid not clear
++	Very big clumps under microscope (granularity just visible to the unaided eye some unagglutinated cells seen under microscope)
+	Not such big clumps (no macroscopic agglutination numerous unagglutinated cells under microscope)
(+)	Less than + clumps of 8–12 cells (many free cells)
w (*weak*)	Weak reactions with uniform distribution of small clumps of 4–6 cells
?	Uneven distribution of cells with no very definite clumps
—	All cells separate and evenly distributed

Some sera possess the property of reacting more strongly with a double dose of the homologous antigen This ability to distinguish the heterozygote is very valuable especially in cases where only the one antibody is available The strength of the reaction is estimated on the titration score according to the amount of agglutination The system of Dunsford and Bowley is given below

C = 12
V = 10
++ = 8
+ = 5
(+) = 3
gw = 2
w = 1

The scores for all tubes are summed and compared with the scores of known cells containing single and double doses of antigens Dosage tests also have other applications since they can be used to indicate whether or not a serum contains more than one antibody

Preparation of apparatus

Special cleaning of apparatus is necessary since agglutinins are absorbed or inactivated by a variety of unrelated materials Washing soda preparations are preferable to detergents which may produce lysis and inhibition of agglutination if not removed entirely from the glassware Tubes which are not sparkling clean when viewed in a good light must be rewashed It is recommended that used precipitin tubes be made up in bundles held by rubber bands and allowed to stay immersed in water overnight They are then placed in a bowl of a strong solution of Brylanz (G Gurr Ltd) ensuring that the tubes are filled, and boiled for 10 minutes After thorough rinsing in tap water they are then transferred to a weak solution (5–10 per cent) of hydrochloric acid for one hour After further thorough rinsing with tap water the tubes are rinsed in distilled water and dried mouth downwards in the hot air oven Blotting paper should cover the bottom of the crates to prevent the tubes falling out

REAGENTS

The reagents used in blood transfusion work are cells soluble antigenic material and sera Some of these are rare and yet must be on hand when needed for which reason they must be capable of storage Soluble antigenic materials such as saliva are stored frozen solid at $-20°$ C in small amounts as also are sera Under these conditions they retain their properties for a considerable time but red cells kept at $+4$ C in dextrose saline lyse after a few weeks and lyse immediately on thawing from the frozen stage However by suspending the cells in a glycerine citrate mixture cells may be frozen solid without damage so that rare genotypes can always be available Dried sera are used occasionally having the advantages of mixing with cells without causing dilution and of being stored at $+4$ C

TECHNIQUE

The following are details of the techniques to which reference is made in this Chapter

agglutinin anti T being present in all adult human sera This is the Hubener Thomsen phenomenon and cells which have been modified by the action of the bacteria are agglutinated by all adult sera The antibody can be specifically removed from sera with the altered cells

Another type of bacteriogenic pan agglutination is caused by sera infected with various skin diphtheroids This causes the sera to develop an anti h agglutinin which reacts with an h antigen on all red cells This must not be confused with the anti H of the ABO system (*see* Chapter 15)

Estimate of agglutination strength

The strength of agglutination is best estimated by using a scoring system and that used at the M R C Blood Group Reference Laboratory is standard in Britain

Grades of agglutination

Grade	Description
C (*complete*)	Clumps of cells in clear surrounding fluid visible to naked eye
V (*visual*)	Rather weaker clumping directly visible but with surrounding fluid not clear
++	Very big clumps under microscope (granularity just visible to the unaided eye some unagglutinated cells seen under microscope)
+	Not such big clumps (no macroscopic agglutination numerous unagglutinated cells under microscope)
(+)	Less than + clumps of 8–12 cells (many free cells)
w (*weak*)	Weak reactions with uniform distribution of small clumps of 4–6 cells
?	Uneven distribution of cells with no very definite clumps
—	All cells separate and evenly distributed

Some sera possess the property of reacting more strongly with a double dose of the homologous antigen This ability to distinguish the heterozygote is very valuable especially in cases where only the one antibody is available The strength of the reaction is estimated on the titration score according to the amount of agglutination The system of Dunsford and Bowley is given below

C = 12
V = 10
++ = 8
+ = 5
(+) = 3
gw = 2
w = 1

CHAPTER 15

THE ABO BLOOD GROUP SYSTEM

GENERAL

HISTORY

In 1900 Landsteiner described the agglutination which occurred when red cells of one individual were exposed to the action of serum from another and so discovered the ABO blood group system Using the naturally occurring antibodies in the serum he was able to identify three blood groups and in 1902 his pupils Decastello and Sturli described a fourth The groups were classified independently by Jansky in 1907 and Moss in 1910, but unfortunately their numerical systems were the reverse of one another To avoid confusion the international nomenclature based on the antigenic content of the cell is used today (Table IX)

TABLE IX
THE LANDSTEINER BLOOD GROUPS (ABO SYSTEM)

Jansky	*Moss*	*International*
4	1	AB
3	2	A
2	3	B
1	4	O

The ABO groups are differentiated by the use of naturally occurring anti A and anti B agglutinins which are normally present in the absence of the corresponding antigen Thus the serum of group A individuals contains anti B sometimes termed *beta* (β) and that of group B individuals contains anti A or *alpha* (α) group AB blood exhibits neither antibody while serum from group O blood possesses both anti A and anti B

In 1911 von Dungern and Hirszfeld described a weaker reacting A antigen which brought the number of groups in the system to six these being A_1 A B A_1B A_2B and O In Britain approximately 20 per cent of A and AB bloods possess the weaker antigen A_2 and

STORAGE OF CELLS IN GLYCEROL

Reagent (*a*) Buffered tripotassium citrate

Tripotassium citrate ($K_3C_6H_5O_7$ H_2O)	32 5 g
Potassium dihydrogen phosphate (KH PO_4)	4 7 g
Dipotassium hydrogen phosphate (K_2HPO_4)	6 0 g
Distilled water to 1 litre	

Reagent (*b*) Laying down solution

Glycerol	40 ml
Buffered tripotassium citrate solution	60 ml

Reagent (*c*) Recovery solutions

(1) Primary 16 per cent solution

Glycerol	25 6 ml
Buffered tripotassium citrate solution	174 4 ml

(2) Prepare 8 4 and 2 per cent solutions by doubling dilution

Reagent (*d*) Anti coagulant (A C D solution)

15 per cent solution anhydrous dextrose	100 ml
2 per cent solution disodium citrate ($Na_2H(C_6H_5O_7)$ H_2O)	500 ml

LAYING DOWN CELLS

(1) Collect venous blood into A C D solution (4 parts of blood to 1 part of anticoagulant)
(2) Centrifuge and remove the supernatant
(3) Gradually add the laying down solution mixing between each addition until an equal volume has been added
(4) Freeze the cells solid at − 20 C

RECOVERY OF FROZEN CELLS

(1) Allow the cell mixture to thaw at room temperature
(2) Centrifuge remove the supernatant and resuspend the cells in an equal volume of 12 per cent aqueous solution of trisodium citrate at room temperature (20 C)
(3) Leave for 10 minutes at this temperature
(4) Centrifuge at 1 000 r p m for 10 minutes
(5) Remove supernatant and wash cells 4–6 times with normal saline or until washings are clear

1952 appeared to be group O in that they were unaffected by anti A and anti B and exhibited the appropriate antibodies, yet they were the bloods of children descended from normal B and O parents and, moreover, the individuals so affected passed the B antigen on to their progeny This phenomenon was explained by Levine and his

(1)

	O	O
A_1	A_1O	A_1O
A_1	A_1O	A_1O

(2)

	A_1	O
A_1	A_1A_1	A_1O
A_2	A_1A_2	A_2O

FIG 40 —(1) The possible progeny from a mating of two homozygotes A_1A_1 and OO results in a typical Mendelian *inheritance that is 100 per cent heterozygotes Using only* anti A and anti B there is apparent dominance of A_1 over O (2) The possible progeny of a mating of two hetero zygotes with a ratio of 3 1 in favour of heterozygotes All however group as A_1 except A_2O which groups as A

associates in 1955 as the effect of modifying genes (the Xx system), the very common modifying gene X being necessary for the proper expression of the B and O genes It is assumed that the very rare allele x in double dose prevents the expression of the genes as anti gens Such a mating would occur only in small communities where consanguinity is likely A similar system of modifying genes (the Yy system) affects the A antigen and may be responsible for the rare A_o bloods Suppression of the effect of genes by other genes is well known in genetics

Human blood chimeras present another problem involving other blood group systems in addition to the ABO blood groups Chimeras of this type are uniovular twins whose red cell forming tissues have become intermixed during the early stages of embryonic life This implies that although each twin will be only capable of passing on their own genotype their bloods will contain cells derived from both mesenchymal element and therefore both sets of antigens The cases reported have been A and O mixtures the true O failing to develop anti A in the presence of the A antigen derived from the mesenchyme of the other twin

2 per cent of A_2 and 25 per cent of A_2B specimens of blood exhibit an antibody active against A_1 cells Normally, sera from group B individuals possess both anti A and anti A_1 capable of agglutinating A_1 and A_2 cells but the ordinary anti A can be removed by absorption with A cells leaving a specific anti A_1 serum Bloods with antigenic properties intermediate between A_1 and A_2 have been described mainly among Negroes

In 1937 Friedenreich described an agglutinogen which was even more feeble than A_2 cells in its reaction with anti A This was designated A_3 the groups A_3 and A_3B being quite rare The agglutination with potent anti A sera is characteristic, consisting of small clumps in a sea of unagglutinated cells The groups almost invariably exhibit anti A_1 behaving as a true cold agglutinin

Another extremely rare type of blood was first described by Fischer and Hahn in 1935 Although unagglutinated by anti A and anti B the cells reacted with O serum ($\alpha + \beta$) Different workers have given different names to the group (A_4 A_5 A A_z and A_o) since it is not clear that they are all the same The group A_5 for example, absorbs anti A but the cells are not agglutinated by O serum

INHERITANCE

In 1924 Bernstein established that the ABO blood group system was inherited in a Mendelian manner He postulated three allelomorphic genes one for each of the antigens A B and O The theory which implied that no child could possess an ABO antigen which was not present in either of the parents blood has been amply proved by a comparison of expected and observed blood groups If further genes for the antigens A_2 and A_3 are postulated the theory is in no way invalidated but merely extended

Testing with anti A and anti B establishes the phenotypes but the genotypes in most cases must be established by pedigree studies Using the available antisera the genotype AO is not recognized neither is BO and it would appear that the gene A_1 is dominant to A_2 A_3 and O The alleles A_2 A_3 and B would also appear to be dominant to the O gene In actual fact if specific antisera were available it would probably be shown that the genes were all co dominants

Two families are illustrated below (Fig 40) to demonstrate the inheritance of the ABO blood group system It will be noted that in blood group genetics antigens and genes are designated by the same symbol

Another rare variant of the ABO system must be mentioned here The Bombay bloods described by Bhande and his co workers in

are derived from animal sources eel serum or serum of goats injected with the somatic antigen of *Shigella shigae* being good sources A very good anti H may be obtained from the seeds of *Lotus tetragonolobus* It is of interest that the Bombay bloods exhibit anti A anti B and a powerful anti H

The literature describes several so-called anti O sera derived from human and animal sources but their properties are not alike They may be differentiated from anti H since they are not inhibited by saliva from ABO secretors The group specific substances present in body fluids provide the material for these inhibition tests since they absorb sera specifically Combined with protein, the group substances may also be used to stimulate the production of high titre antisera in volunteers

PLANT HAEMAGGLUTININS

Haemagglutinins derived from plants were first described in 1888 by Stillmark and have since been shown to be valuable research tools The agglutinins found principally in seeds are often specific and may behave as red cell agglutinins or as precipitins with antigenic material such as saliva

The seed of *Dolichos biflorus* is mainly A_1 specific and is therefore of great value in the differentiation of the subgroups of A Bird and Morgan in 1958 showed that a purified dolichos extract used as a precipitin made a sharp distinction between A_1 and A_2 salivas Other seed extracts are specific with saline suspensions of A cells but cross react with B and O bloods in protein media

No specific agglutinin for B cells has yet been discovered but mixtures of anti A and anti B are relatively common *Crotalaria stricta* and usraemonensis extracts provide such mixtures which are more active against A than B cells while *Sophara japonica* extracts agglutinate B cells more than A cells

Seed anti H agglutinins from *Lotus tetragonolobus* and *Ulex europaeus* are extremely useful since they react better with the A subgroups and also distinguish between secretor and non secretor saliva

Anti N agglutinins have been described in the seeds of *Vicia graminae* and other plants The extracts cross react weakly with M cells but this is also a fault of some antisera and may be eliminated by dilution

THE DIFFERENTIATION OF A SUBGROUPS

The A antigen varies in its reaction with antisera which recognize A_1 and the weaker A and A_3 antigens Although the differentiation

THE SECRETION PHENOMENON

About 78 per cent of white people possess water soluble forms of the ABO antigens demonstrated in body fluids, such as saliva tears sweat semen and gastric juice The water soluble form of the antigen is a hapten which being devoid of protein is non antigenic and is termed a group specific substance Individuals in whose body fluids the substances can be demonstrated are termed secretors and the remainder non secretors It has been suggested that the secretion phenomenon is yet another manifestation of a modifying gene termed Se the allele se in double dose resulting in failure to form the water soluble substances The genes segregate independently of the ABO and other blood group genes but secretion is not confined to the ABO system the Lewis system (*see* Chapter 18) also being involved

ANTIGENS AND ANTIBODIES

The A and B antigens can be detected in the early embryo and increase in strength as judged by agglutinability from birth to puberty the reaction then tending to get weaker as age advances The naturally occurring antibodies are not detectable at birth any anti bodies present being derived from the maternal circulation by passive transference The ABO antibodies may take up to 6 months to develop and normally increase in strength and diminish in old age like the antigens

Immune anti A and anti B have a reversed thermal optimum that is they react better at 37 C and they sometimes exhibit zoning and a blocking effect The immune antibody can fix complement and also can be identified by partial neutralization with A or B substance If increasing amounts of A substance are added to immune anti A serum the haemolytic property disappears first before the power to agglutinate cells in saline Although the power of agglutinating cells in saline also disappears on partial neutraliza tion the serum can still agglutinate cells in serum or albumin

A true anti O serum has not been described the O gene being generally accepted as an amorph Group O cells however are agglutinated by anti H serum H substance seeming to be a common antigen found in the body fluids of all ABO secretors The amount of H substance present seems to be greatest in group O individuals and least in the fully mutated A_1B person The exact relationship between O and H antigens is unknown Anti H occurs naturally in individuals lacking the antigen and may be called into being by the injection of group O cells into such a person Most potent antisera

of the subgroups of A is mainly of academic interest, it is of import ance in blood transfusion since the subgroups may exhibit an *alpha* agglutinin of high titre The techniques used to differentiate the subgroups are many and varied including cell reactions with naturally occurring and immune antibodies with extracts of seeds and with partially absorbed and fully absorbed sera The use of serum from a group O individual to pick up the weaker A antigens is capable of two alternative explanations It is postulated that a third antigen C exists individuals who are A, B and AB possessing this antigen and O serum containing the corresponding antibody Such an antibody would enhance the agglutination of weak A cells, but the theory is not accepted by most authorities The second explanation, that *alpha* and *beta* antibodies are present on the same molecule in group O serum, seems to be proved by the fact that A cells exposed to O serum ($\alpha + \beta$) and then washed, can be shown to have absorbed both *alpha* and *beta* agglutinins although the amount of anti B is small Similarly B cells after similar treatment can be shown to have absorbed a small amount of *alpha*

RELATIONSHIP TO DISEASE

Statistically there would appear to be a relationship between the ABO blood group system and certain diseases Group A individuals are more likely to suffer from gastric carcinoma than group O individuals who however, have a greater tendency to duodenal ulcer Pernicious anaemia seems to occur more in Group A persons and an association between this group and diabetes mellitus seems probable It is conceivable that liability to many diseases is genetic ally determined the genes showing linkage with the blood group genes

MEDICO LEGAL ASPECTS

Since the inheritance of the ABO blood group system rests on such sure ground it would seem that paternity disputes could in many cases be settled by reference to a blood group laboratory The subgroups impose some limitations on the value of the procedure since it is not possible to distinguish such a heterozygote as A_1A_2 from the homozygote A_1A_1 Moreover some authorities would believe that the observed but unexpected appearance of a heterozygous subgroup should not necessarily be conclusive evidence assuming that it might be produced in some way from a union o non subgroups

TABLE X

THE DIFFERENTIATION OF THE SUBGROUPS OF A

	Anti A	*Anti A_1*	*O serum*	*Anti H*	*Immune anti A*	*O serum absorbed with B substance*	*Immune anti A + Indirect Coombs test*	*Extract of* Dolichos bifloros *seeds*	*Extract of* Phaseolus lunatus *seeds*	*Agglutinins present in serum*
A_1	V	V	V	W or Neg	V	V	+	V	V	Anti H (rare)
A_2	V or ++	Neg	V or ++	++	V	V or ++	Neg	++	++	Anti A_1 (often)
A_3	W (free cells)	Neg	+ (free cells)	++	++	+	Neg	Neg	+	Anti A_1 cold
A_o	W or Neg	Neg	+	V	[illegible]	W or Neg	Neg	Neg	Neg	Anti A_1 cold
O	Neg	Neg	Neg	V	Neg	Neg	Neg	Neg	Neg	$a + \beta$ or Anti A + Anti B

TUBE TITRATION

(1) Collect venous blood into a dry sterile bottle and allow to clot
(2) Allow the clot and serum to remain in contact at 4 C overnight to remove cold agglutinins
(3) Remove serum in the cold and inactivate complement at 56 C for 30 minutes
(4) Make doubling dilutions of the serum in saline starting at 1 2
(5) Add an equal volume of 5 per cent suspension of appropriate cells to each tube and mix
(6) Incubate at room temperature for 1 hour
(7) Read macroscopically and microscopically recording the titre as the reciprocal of the greatest serum dilution showing no macroscopic agglutination but small clumps of cells microscopically with numerous free cells (+ reaction) Control the test with cells of the other ABO groups

Note All tube titrations should be set up in duplicate

PREPARATION OF ANTI A_1 SERUM

(1) Remove all traces of plasma from fresh A cells by washing three times with large volumes of saline at room temperature
(2) Mix 1 volume of a suitable high titre anti A serum with one third to one quarter of its volume with washed packed A cells
(3) Stand at 4 C overnight
(4) Centrifuge remove the supernatant serum and titrate against A_1 and A_2 cells

Note (*a*) If the serum after absorption is specific or can be made specific by dilution for A_1 cells determine the optimum dilution for economic use by titration against A_1B cells
Note (*b*) If the absorption is insufficient as shown by no distinction between A_1 and A_2 cells being possible repeat with fresh A cells
Note (*c*) Not all anti A sera are suitable for absorption
Note (*d*) All absorbed sera tend to lose specificity on storing as absorbed agglutinins reappear in low titre so all tests should be controlled with A_1B and A_2 cells

PREPARATION OF ANTI H SERUM

(1) Soak seeds of *Lotus tetragonolobus* in an excess of distilled water for 12–18 hours at room temperature
(2) Decant the remaining water and macerate the seeds
(3) Add 3 volumes of 0 85 per cent saline to 1 volume of macerated seeds and leave 1 hour at room temperature
(4) Centrifuge and remove the supernatant
(5) Filter the supernatant through Whatman No 1 filter paper and store in small quantities frozen solid at − 20 C

Forensic work

The identification of blood groups from dried stains is an accepted procedure in forensic work as is the recognition of blood groups by the substances present in saliva or semen Absorption techniques are used throughout, but the reactions are inhibited by some materials used in dressing cloth urine nasal secretion and so on Should the suspect material be from a non secretor it is not possible to decide the blood group from body fluids

TECHNIQUE

The following are details of the techniques relevant to tests mentioned in the earlier part of this chapter

SELECTION OF ANTISERA

Anti A (α) is provided by a group B donor
Anti B (β) is provided by a group A donor
O serum (α + β) is provided by a group O donor

TEST FOR AVIDITY

(1) Take an equal volume of capillary blood directly into 3 8 per cent sodium citrate and centrifuge immediately
(2) To 1 drop of the supernatant on a white tile add an equal volume of 20 per cent suspension of A_1 or B cells prepared from washed packed cells
(3) Start a stop watch and rock the tile gently

If macroscopic agglutination shown by a graininess appears within 35 seconds then serum from the donor will react powerfully at a dilution of 1 4 and is generally suitable as a high titre grouping serum

SCREENING TEST FOR HIGH TITRE DONORS

(1) Cut a strip 2 inches wide of Ford s white blotting paper (428 mill) and place across a Petri dish lid so that the centre is unsupported
(2) With a pipette delivering 25 drops per ml place 2 drops of undiluted serum on the blotting paper and allow to spread
(3) With a pipette delivering 40 drops per ml held 5–6 mm above the centre of the serum drop add 1 drop of the appropriate 5 per cent cell suspension and allow to spread for 30–45 seconds
(4) Assist the spreading of the cell suspension by dropping 2 drops of saline at intervals of 2–5 seconds in the centre of the serum-cell mixture using the larger pipette Control the test with a known potent antiserum against the same cells

The less the extent of spread by the cell suspension the higher the antibody titre Sera comparing favourably with the controls are tested by a tube titration

(4) To column A in block I add 1 drop of A_1 cells 5 per cent suspension to column B 1 drop of B cell suspension and to the others 1 drop of the patient s cell suspensions
(5) Into the first column of tubes in block II pipette 2 drops of the first patient s serum and repeat with the sera from the other patients
(6) To the first row of tubes in block II add 1 drop of 5 per cent suspen sion of A_1 cells to the second row add 1 drop of B cell suspension to the third 1 drop of O cells and to the back row 1 drop of cells from the appropriate patient
(7) Mix by flicking each tube with the finger cap and allow to stand 2 hours at room temperature
(8) Read macroscopically and microscopically

This technique should pick up the lower grades of the A antigen and differentiate them The auto control in the serum agglutinin block *eliminates pan agglutination errors*

DETERMINATION OF SECRETOR STATUS

(1) Rinse mouth of donor with water
(2) Collect a minimum of 0 5 ml saliva With babies collect saliva on a cotton wool swab and squeeze saliva into a tube
(3) Heat saliva in boiling water bath for 10–20 minutes to destroy inactivating enzymes
(4) Centrifuge for 5 minutes at 2 000 r p m and pipette off the opales cent supernatant
(5) Store supernatant frozen solid at − 20 C until required
(6) Dilute saliva 1 2 with saline and add to an equal volume of diluted anti serum (anti A anti B or anti H) to titre of 1 8 or to give just good visual agglutination with the appropriate cells
(7) Leave at room temperature 30 minutes
(8) Add 1 volume of 2 per cent suspension of appropriate group red cells and leave at room temperature 1½–2 hours
(9) Read results microscopically Inhibition of agglutination indicates that the saliva is from a secretor

Note (*a*) *Control with 1 volume of diluted antiserum and 1 volume* of saline
Note (*b*) With anti A use A_2 cells as a more sensitive indicator

TITRATION OF SALIVA

(1) Make doubling dilutions of saliva to five tubes
(2) Into each tube place 1 volume of diluted antiserum (*see* above)
(3) Allow to stand at room temperature 30 minutes
(4) Into each tube place 1 volume of the appropriate cell suspension mix and allow to stand 1–2 hours at room temperature

ABO GROUPING—TILE METHOD

(1) Mark off a clear white tile in squares with a grease pencil and label the first horizontal row anti A and the second anti B Mark the first vertical column A cells and the second B cells Subsequent vertical columns bear the names of patients
(2) Place 1 drop of anti A in each square of the first horizontal row and 1 drop of anti B in the squares of the second horizontal row
(3) Place 1 drop of 5 per cent suspension of A_2 cells in the two squares of the first vertical column and 1 drop of a similar suspension of B cells in the squares of the second vertical column These constitute known agglutinogen controls
(4) Place the patient s cell suspension in the squares allocated and gently rock the tile
(5) Read macroscopically after 3–5 minutes

The patient s serum is tested similarly for agglutinins using A_1 and B cells but if they are weak the preparation may dry up before agglutination is obvious

ABO GROUPING WITH THE MURRAY SLIDE

(1) On the first platform of a Murray slide place a drop of anti A serum and on the second a drop of anti B serum Miss one space and repeat the procedure for as many groups as are being tested and two controls
(2) On the first two platforms place 1 drop of 5 per cent suspension of A_1 cells on the second pair of platforms 1 drop of similar suspension of B cells and on subsequent pairs cell suspensions of the patients in alphabetical order
(3) Breathe on the slide and rock to mix the reagents
(4) Place in a moist chamber at room temperature for 15–30 minutes
(5) Read macroscopically and microscopically

Serum agglutinins are determined in a similar manner

It is recommended that tile or slide techniques always be confirmed by a tube technique

ABO GROUPING—TUBE TECHNIQUE

(1) Place four rows of precipitin tubes in a wooden block and label each column of four with a paper flag placed in the fifth hole Label the first two columns A and B and subsequent columns with the names of the patients
(2) Place a second series of tubes in another block and label similarly omitting A and B
(3) Into the first row of tubes in block I pipette 1 drop of anti A into the second row 1 drop of anti B into the third row 1 drop of O serum and into the back row 1 drop of anti A_1 serum

The antiserum used in the next part of the test is that which has had the least amount of saliva added and no evidence of residual saline anti A

(1) To 2 volumes of incomplete anti A neutralized as above in a small tube add 1 volume of test cells in saline 50 per cent suspension
(2) Incubate 2 hours at 37 C
(3) Wash the cells three times with large volumes of saline
(4) Perform Coombs test on final packed cells
Control with standard A_1 and A_2 and O cells similarly treated

The Coombs serum must be diluted to optimum titre for such sensitized cells Varying dilutions may need to be tried

INVESTIGATION OF A SERUM FOR α or β HAEMOLYSINS

(1) Make doubling dilutions of the serum in saline
(2) Add an equal volume of fresh human serum (free of A or B substance and α or β haemolysins) as a source of complement
(3) Add an equal volume of 5 per cent suspension of cells of appropriate group
(4) Tap to mix cap and incubate 1 hour at 37° C

Note (*a*) Control non specific lysins with the unknown serum + complement + O cells
Note (*b*) Control complement with A_1 or B cells to show that it is lysin free
Note (*c*) Serum from group A or B individuals may contain free A or B substance in quantity sufficient to neutralize a weak lysin Therefore serum from a group B individual is unsuitable as a source of complement when testing for β lysins and serum from Group A individuals is unsuitable when testing for α lysins

DIFFERENTIATION BETWEEN ANTI H AND ANTI O

(1) To 1 volume of the serum add 1 volume of saliva from a known ABO secretor Group O secretors are most reliable for this purpose since those of group A or B are not always strong secretors
(2) Leave at room temperature for 1 hour
(3) Add an equal volume of 2 per cent suspension of Group O cells to each tube leave 2 hours at room temperature and read

Note (*a*) Control with 1 volume of serum and 1 volume of saline
Note (*b*) The antibody is anti H if its action is inhibited by saliva but a mixed Lewis antibody (*see* Chapter 18) is also inhibited
Note (*c*) The antibody is anti O if it is not inhibited by saliva but most antibodies reacting with high frequency antigens (*see* Chapter 18) also agglutinate O cells and are not inhibited

(5) Read microscopically and report titre as that tube in which inhibition is complete

Note (a) Control with diluted serum and saline

Note (b) The test may be altered by titrating the serum and adding a constant amount of saliva to each tube but this is not a measure of the group substance

ABO SUBGROUPING—SCORING OF TITRATION RESULTS WITH ANTI A O SERUM ANTI H, IMMUNE ANTI A

(1) Make doubling dilutions of the antisera in triplicate

(2) To the tubes of one set add 1 volume of 2 per cent suspension of test cells to the second set add 2 per cent suspension of known (standard) A_1 cells and to the third set add a similar suspension of known (standard) A_2 cells

(3) Incubate for 2 hours at room temperature for anti A O serum and anti H and 2 hours at 37 C for the immune anti A

(4) Read scoring each tube numerically and total the scores for each titration

USING O SERUM ABSORBED WITH B CELLS

(1) To O serum add 1 4–1 3 volume of washed packed B cells

(2) Allow to stand overnight at 4 C

(3) Centrifuge and take off serum

(4) Test against B cells to ensure absorption is complete

(5) Titrate the serum and score as in previous technique using the standard cells for comparison

USING O SERUM NEUTRALIZED WITH B SUBSTANCE

(1) To O serum add an equal volume of boiled saliva from a B secretor

(2) Allow to stand at 4 C for 1–2 hours

(3) Treat as serum titrate and score as in previous techniques

USING INCOMPLETE ANTI A

Note Incomplete anti A serum always contains saline agglutinins which must be neutralized since they will cause false positives in this test As a corollary *immune anti A or anti B may be detected in the same manner* by neutralizing saline antibody and subsequently testing for the ability of the serum to sensitize standard cells

(1) Place 0 5 ml of the incomplete anti A serum in each of three tubes

(2) To the first tube add 0 5 ml to the second 1 0 ml and to the third 1 5 ml of boiled saliva from a Group A secretor

(3) Leave at room temperature for 1 hour

(4) Pipette 0 02 ml of the mixtures into separate precipitin tubes and to each add 0 02 ml of a 2 per cent suspension of fresh A_1B cells in saline

(5) Leave for 1 hour at room temperature and read

The antiserum used in the next part of the test is that which has had the least amount of saliva added and no evidence of residual saline anti A

(1) To 2 volumes of incomplete anti A neutralized as above in a small tube add 1 volume of test cells in saline 50 per cent suspension
(2) Incubate 2 hours at 37 C
(3) Wash the cells three times with large volumes of saline
(4) Perform Coombs test on final packed cells
Control with standard A_1 and A_2 and O cells similarly treated

The Coombs serum must be diluted to optimum titre for such sensitized cells Varying dilutions may need to be tried

INVESTIGATION OF A SERUM FOR α or β HAEMOLYSINS

(1) Make doubling dilutions of the serum in saline
(2) Add an equal volume of fresh human serum (free of A or B substance and α or β haemolysins) as a source of complement
(3) Add an equal volume of 5 per cent suspension of cells of appropriate group
(4) Tap to mix cap and incubate 1 hour at 37 C

Note (*a*) Control non specific lysins with the unknown serum + complement + O cells
Note (*b*) Control complement with A_1 or B cells to show that it is lysin free
Note (*c*) Serum from group A or B individuals may contain free A or B substance in quantity sufficient to neutralize a weak lysin Therefore serum from a group B individual is unsuitable as a source of complement when testing for β lysins and serum from Group A individuals is unsuitable when testing for α lysins

DIFFERENTIATION BETWEEN ANTI H AND ANTI O

(1) To 1 volume of the serum add 1 volume of saliva from a known ABO secretor Group O secretors are most reliable for this purpose since those of group A or B are not always strong secretors
(2) Leave at room temperature for 1 hour
(3) Add an equal volume of 2 per cent suspension of Group O cells to each tube leave 2 hours at room temperature and read

Note (*a*) Control with 1 volume of serum and 1 volume of saline
Note (*b*) The antibody is anti H if its action is inhibited by saliva but a mixed Lewis antibody (*see* Chapter 18) is also inhibited
Note (*c*) The antibody is anti O if it is not inhibited by saliva but most antibodies reacting with high frequency antigens (*see* Chapter 18) also agglutinate O cells and are not inhibited

CHAPTER 16

THE MNSs AND P BLOOD GROUP SYSTEMS

GENERAL

HISTORY

In 1927–28 Landsteiner and Levine described the MN blood groups and postulated that the antigens were inherited by means of two allelomorphic genes without dominance producing three genotypes In Britain the genotypes have the following frequencies MM 28 per cent MN 50 per cent and NN 22 per cent The mode of inheritance was confirmed by pedigree studies and the discovery of weaker agglutinogens explained by further allelomorphs In 1947 Walsh and Montgomery described another antibody related to the MN system The reactions were investigated by Race and Sanger and the antibody was termed anti S An antibody giving antithetical reactions has since been described by Levine thus confirming the hypothesis that S and s form another pair of genes closely linked with M and N The linkage must be very close since crossing over has not been shown to occur

THE MN ANTIGENS

The MN antigens do not easily provoke antibody formation in man but do so in some animals so diagnostic sera are usually produced in rabbits The subgroup N is a weaker antigen not at all affected by some anti N sera and a weaker M antigen has been described An intermediate form having characteristics between M and N is termed M^c

THE MN ANTIBODIES

Naturally occurring anti M and anti N reacting best at 4 C have been described but are rare As a result of transfusion immunization antibodies having a thermal optimum of 37 C may be produced in susceptible individuals and have occasionally been reported in the sera of pregnant women The antibodies produced in rabbits usually react best at 18–20 C but the means of production is not a sure guide to the thermal optimum A simple tile technique may be used to MN type red cells agglutination usually being rapid

and intense Dried antisera which merely need stirring into the red-cell suspension, are commercially available

THE Ss ANTIGENS

The Ss antigens are potent in man and have caused both trans fusion reactions and haemolytic disease (*see* Chapter 17) The antigen S is linked more with M than N in Europeans The four possible chromosomes of the MNSs system give rise to 10 possible genotypes shown in Table XI Using the three available sera anti M, anti N and anti S the presence of the antigen s is assumed in the absence of S Of the British people 55 per cent are S positive

THE Ss ANTIBODIES

Naturally occurring anti S reacting at 37° C has been described, but most sera are derived from transfusion immunization or cases of haemolytic disease Anti s is a rarity but since anti S in common with other antibodies of the group, possesses dosage properties it is not really needed for typing An antibody with dosage properties reacts much better with homozygous than heterozygous cells Anti S occurs often as an immune antibody in contrast with anti M and anti N which are nearly always saline antibodies Anti S has caused haemolytic disease and has been deliberately produced by immunizing volunteers Although the reacting temperature of the antibodies varies most anti S sera have a thermal optimum of 37° C as do the few anti s sera It may be necessary to employ the anti globulin reaction to demonstrate the presence of the anti bodies

ASSOCIATED ANTIGENS

Certain blood group antigens of low incidence are associated with the MNSs group system The factors Mi^a (Miltenberger) and Vw (Verweyst) are related to one another and inherited with the MNSs system Vw travelling with Ns Although the antigens are not common anti Mi^a is of more frequent occurrence and has been found as a naturally occurring antibody

Two antigens termed Hunter and Henshaw related to the MNSs system have been described in Negroes They appear to be inherited as Mendelian dominants and are not obvious allelomorphs A further very common antigen termed U is also related and it is suggested that U negative cells should be called S^uS^u, implying that it is an allelomorph of S and s

It will be seen from these considerations that the MNSs blood group system is one of great complexity with many problems still to be investigated

TABLE XI

THE MNSs BLOOD GROUP SYSTEM

Phenotype	*Possible genotypes*
MS	MSMS or MSMs
Ms	MsMs
MNS	MSNS or MSNs or MsNS
MsNs	MsNs
NS	NSNS or NSNs
Ns	NsNs

THE P BLOOD GROUP

At the same time that they were elucidating the MN system Landsteiner and Levine described another blood group unrelated to the ABO system The P antigen is inherited as a Mendelian dominant and appears in graded strengths according to the geno type The strength of the reaction indicates individuals who are P_1 P_2 and p (P negative) but potent antisera might react with indi viduals apparently negative with other sera In Britain about 80 per cent of people are P positive

A further antigen belonging to the P system has been described A specific antibody exists for this antigen which is designated P^k but the gene does not express itself in the presence of P_1 and P_2

THE P ANTIBODY

The agglutinin anti P occurs commonly as a naturally occurring antibody in P negative individuals and reacts as a cold agglutinin The pig and the horse often possess anti P of good titre in their serum and are a good source of supply Immune anti P capable of haemolysing P positive cells at 37 C has been described as produced by transfusion immunization and anti P in the blood of P negative pregnant women is very common Transfusion reactions however are uncommon and there is no evidence of the association of anti P with haemolytic disease

ASSOCIATED ANTIGENS

There is a definite relationship between the P blood group system and the Jay system The antigen Tj^a is found on almost all red cells

and the antibody anti Tj^a is probably naturally occurring, but the antisera consist of a mixture of anti P and an antibody reacting with all P_1 P and p cells except those of p individuals who are Tj^a negative and whose sera contain anti Tj^a

MEDICO LEGAL ASPECTS

The MNSs blood group system has been used in medico legal work and is of value in cases of doubtful paternity Together with the ABO system blood tests " become a potent factor in such cases

TECHNIQUE

The following are details of techniques allied to data of the first part of this chapter

PROVISION OF ANTISERA

Rabbit anti-M and anti N

(1) Select six rabbits on a full protein diet for each antiserum
(2) Take blood into acid-citrate dextrose solution from OMM and ONN *donors*
(3) Wash the cells at least three times with saline to remove all traces of plasma
(4) Give 0 5 ml of washed packed OMM cells intraperitoneally to six rabbits and the same amount of ONN cells to the other six animals
(5) On the next day and the following 4 days give each animal 0 5 ml of the appropriate packed cells intravenously
(6) 10–14 days after the last injection bleed each animal for 30–40 ml from the ear vein
(7) Allow blood to clot overnight at 20 C
(8) Separate serum and heat at 56 C for 30 minutes to destroy com plement

The naturally occurring anti A and human species agglutinins must be removed from the rabbit serum For anti M sera use A_2NN cells and for anti N use A_1MM cells both washed free of plasma

(1) Prepare two sets of tubes each of 2 ml of serum diluted with saline 1 10 1 20 and 1 40 respectively
(2) To one of the 1 10 dilutions add 0 5 ml of washed packed A_1 cells and to the other 1 ml Mix and leave 4–6 hours at 18 C
(3) Treat the other four tubes of diluted serum in a similar manner
(4) Gently mix centrifuge and remove supernatant serum
(5) Titrate each supernatant against A_1MM A_1MN and A_1NN cells at 18 C The serum which gives a positive reaction at 1 8 with MN cells and little or no reaction with the negative control indicates the suitable combination of dilution factor and volume of cells for optimal absorption

(6) Dilute the bulk serum as indicated and add the requisite amount of washed packed cells Absorb 4–6 hours at 18° C
(7) After absorption titrate with MM MN and NN cells at 18 C To ensure absorption complete and obtain optimal titre

Note Ensure that the serum is free of anti B agglutinins since occasionally rabbits have a naturally occurring anti B
Store in small amounts frozen solid at — 20 C

(*B*) *Anti P serum*

(1) Inactivate pig or horse serum at 56 C for 30 minutes
(2) Dilute the serum with an equal volume of saline
(3) Mix the diluted serum with an equal volume of washed packed P negative cells of groups A B and O to remove unwanted agglutinins
(4) Allow to stand overnight at 4 C and separate the serum at this temperature
(5) Test the serum against P negative cells of all ABO groups to ensure that absorption is complete
(6) Titrate against P_1 and P_2 cells using control of P negative cells Select sera which give clear-cut reactions with P_1 and P negative cells and have a titre of 1 8 or more with P cells The titrations may be left at 20 C for 2 hours or overnight at 4 C

MNSs AND P TYPING

Using anti M and anti N sera

(1) Place 1 drop of appropriately diluted antiserum on an opal tile and add an equal volume of a 5 per cent suspension of cells
(2) Rock the tile and observe for agglutination Use OMN cells as a positive control and the appropriate cells as negative Absorption controls are necessary since absorbed antibodies tend to creep back in stored sera It is also necessary to use more than one antiserum to detect variants which react with one antiserum but not with another

The tile technique given above is convenient but is not recommended for MN typing In any event results obtained by tile techniques should always be confirmed by a tube method

(1) Dilute three different anti M and anti N sera to the optimal strength to give no reactions with fresh ONN and OMM cells respectively
(2) Place 0 03 ml of antisera in precipitin tubes and add 0 03 ml of 2 per cent suspension of test cells to each
(3) Control each serum with fresh OM ON and OMN cells
(4) Leave at room temperature for 2 hours
(5) Gently flick the tubes with the finger and take off the cell suspension
(6) Examine microscopically discarding results obtained with a serum giving more than a weak reaction with the negative control

Using anti S serum

(1) Place 1 volume of antiserum diluted to optimum titre in a precipitin tube and add an equal volume of 2–5 per cent saline suspension of washed cells

(2) Incubate 2 hours at 37° C and read microscopically Control with known S positive and S negative cells and absorption control of A_1BS negative cells

Note Anti S is a rare serum Use small volumes

Using anti P serum

(1) Place 1 volume of anti P serum in a precipitin tube and add an equal volume of 2–5 per cent suspension of washed red cells

(2) Leave at 20 C for 2 hours or overnight at 4 C depending on thermal optimum of serum and read microscopically on chilled slides Control with P_1 P and P negative cells and A_1BP negative cells to ensure absorption is complete

CHAPTER 17

THE Rh BLOOD GROUP SYSTEM

GENERAL

HISTORY

In 1940 Landsteiner and Wiener injected red cells of the monkey Macacus rhesus into guinea pigs and rabbits, stimulating the pro duction of an antibody active against the monkey cells The anti body also agglutinated the cells of 85 per cent of the white popula tion tested and since such individuals obviously possessed the monkey antigen they were termed rhesus positive and the remainder rhesus negative For clinical purposes this distinction is still made today

This was not the first description of a rhesus antibody since Levine and Stetson had already reported in 1939 the discovery of an atypical antibody in the serum of a woman who had been delivered of a stillborn child The antibody which was active at 37 C agglutinated the red cells of 83 of 104 group O donors tested and was shown to be independent of other blood group systems

The two antibodies were shown to be identical by Wiener and Peters in 1940 and a year later Levine and his colleagues demon strated that blood group incompatibility between mother and child could result in a haemolytic disorder in the infant This also was not a new idea foetal immunization of the mother having been suggested by Dienst in 1905 and again by Ottenberg in 1923 as being respon sible for some disorders of pregnancy Darrow in 1938 even suggested that haemolytic disease of the newborn resulted from foetal immunization of the mother avoiding blaming the red cells directly by suggesting that the causative agent was the red cells or some component of them

INHERITANCE

The present day theory of the nature of the rhesus blood group system is due to Sir Ronald Fisher who postulated six antigens inherited by closely linked pairs of allelomorphic genes The discovery of another antibody raised the number to four pairs and subgroups have added to this number The main genes and antigens

are designated C D, E, F and c, d, e, f, a capital and a small letter indicating a pair of alleles Only one of each pair can be carried on a chromosome the possible number of chromosomes with pairs of genes being calculated from the square rule, that is, $2 \times 2 \times 2 \times 2$ (four pairs) = 16 A number of these chromosomes will be rare and some will appear in matings of heterozygotes when they are not expected This is due to crossing over which, however is relatively infrequent so that it is assumed that linkage is very close

The subgroups are inherited by separate allelomorphs and when estimating total genotypes must be calculated separately and added to those of the main genes A third gene allelomorphic to C and c is termed C^w, a fourth C^u a fifth C^x and yet another c^v Third and fourth allelomorphs at the E^e locus are known as E^u and E^w

RHESUS ANTIGENS

The original rhesus antibody recognized the antigen D in those individuals who were Rhesus positive The antigen D is the most potent of the rhesus antigens and probably C is the next potent but individuals vary greatly in their ability to make antibodies Theoretically an individual lacking three of the antigens can make antibodies against them but although combinations of antibodies occur, most cases of rhesus immunization are due to the antigen D This antigen exists in various grades of strength from the low grade subgroup D^u to a stronger reacting form Most D antigens are inter mediate between these two Cells giving some but not all the reac tions expected of D are termed D^u and presumed to be of genotype D d, the homozygote being very rare in Britain and the available sera not recognizing D^uD The D^u antigen is recognized by its behaviour with different anti D sera, the very lowest grades only being detected by the use of the strongest incomplete antisera followed by Coombs test The higher grade D^u cells are agglu tinated by some but not all saline anti D sera The antigen D^u is potent and in the absence of D can stimulate the production of anti D a D^u individual can also make anti D It is possible that 30 per cent of cells apparently Cde/cde are really CD^ue/cde and 10 per cent of apparent cdE/cde are really cD^uE/cde A rare form of the rhesus antigen exists in which owing to a deletion of part of the chromosome, all the available antigenic material is used by D In such cases the amount of D antigen is so great the cells are agglutinated in saline suspension by incomplete anti D sera The genotype is written -D-/-D- and such an individual is capable of

making multiple antibodies the genotype being originally recognized by this phenomenon

The antigens C and C^w are equally potent but immunization with C often produces anti C + anti C^w The c^v antigen is so called because although it reacts with some anti c and anti C sera c c cells give stronger reactions than c^vC cells C^x gives some but not all of the reactions expected of C with pure anti C and cells containing the antigen C^u are affected by anti C without agglutination

The e antigen is present in the cells of 98 per cent of the British population hence immunization is uncommon but the auto anti body is found in some haemolytic anaemias

The f antigen is associated with the antigens cde and cDe in these combinations on the chromosome The antigen F cannot be recog nized except as the absence of f

The c antigen is quite potent but d would appear to be a very weak antigen indeed

The rhesus antigens are not secreted in the body fluids but may be detected in extracts of the organs

RHESUS ANTIBODIES

Naturally occurring antibodies of the rhesus system have been described especially anti E Immune antibodies are found in heterospecific pregnancies and transfusion immunization, and may be deliberately made by immunization of volunteers Albumin antibodies are common with this group indeed it is rare to find a pure saline antibody without some trace of albumin antibody Mixtures are common anti E + anti c anti C + anti D and so on, as might be expected from the genotypes of this group Pure anti C anti D and anti C^w anti E and anti c have all been described but anti e is quite rare and anti f even more rare No specific anti D exists neither does anti C^u and anti c A specific anti E^w is known but anti E^u does not exist

Many of the rhesus antibodies show a dosage effect in that they react better with the cells of the homozygote This is very useful in genotyping particularly if the alternate antibody is not available Thus E/E cells can be differentiated from E/e without the use of anti e if anti E serum with dosage properties is available Anti C anti C^w anti c anti E anti e and anti f have dosage properties some of them quite marked Anti D has no real dosage properties

Some rhesus antisera have another effect in that they can dis tinguish between genotypes such as CDe/cDE and CDe/cde in that the latter reacts better with anti C sera It is known that a double

dose of D has a depressing effect on C and knowledge of such serological properties is invaluable in genotyping

NOMENCLATURE

The Fisher nomenclature describes the antigens present but is cumbersome in speech Shorthand notations are easier to use but the Wiener notation although being different for phenotypes and genotypes changes from time to time and has not yet reached stability

TABLE XII

THE Rh SYSTEM NOMENCLATURE

The Rh chromosomes			*The Rh antibodies*	
Fisher	*Short symbol*	*Wiener*	*Fisher*	*Wiener*
CDe	R_1	R^1	anti C	anti rh
cde	r	r	anti c	anti hr
cDE	R_2	R^2	anti D	anti Rh_0
cDe	R_0	R^0	anti d	anti Hr_0
Cde	R	r	anti E	anti rh
cdE	R	r	anti e	anti hr
CDE	R_z	R^z	anti C^w	anti rh^w
CdE	R_y	r^y		
C^wde	R w	r or r^w		
C^wDe	R_1^w	R^1 or R^w		

RHESUS GENOTYPING

The rhesus group is determined using anti D serum thus dividing individuals into Rh positive and Rh negative A better term for the procedure is D typing and this is done in all cases needing transfusion and in pregnancy since it is possible to produce damaging antibodies under such conditions It is necessary to genotype all rhesus negative individuals since such donors should be cde/cde Using only anti D serum a donor of genotype Cde/cde would be termed rhesus negative but would be a dangerous donor for a recipient cde/cde since the C antigen is very potent

Rhesus genotyping using all the sera gives complete results but in most laboratories only four antisera are available and one or more of these may be of the albumin type The sera must be controlled with positive and negative cells the positive being of cells which will give a weak reaction in order to detect minor deteriorations in the antisera For negative controls cells which can detect serological contaminants are chosen Such contaminants may

be traces of other rhesus antibodies or ABO or other antibody which has been inadequately absorbed To cover these eventualities two sets of controls are needed one for the ABO system and the other for the rhesus system

TABLE XIII

Rh GENOTYPING CONTROLS

Antiserum	*Positive control*	*Negative control*	*Absorption control*
Anti c	CDe/cde (R_1r)	CDe/CDe (R_1R_1)	A_1B R_1R_1
Anti E	CDe/cDE (R_1R_2)	CDe/CDe (R_1R_1)	A_1B R_1R_1
Anti D	CDe/cde (R_1r)	Cde/cde (R r)[1]	A_1B r r
Anti C	CDe/cDE (R_1R_2)	cDE/cDE (R_2R_2)	A_1B r r
Anti-e	CDe/cDE (R_1R_2)	cDE/cDE (R_2R_2)	A_1B R_2R_2

cde/cde (rr) is a more easily obtained alternative

Where genotypes cannot be assumed from the phenotypes using only four sera dosage tests may be invaluable but controls are very essential in such examinations

Results of testing with the rhesus antisera are scored in the manner described in Chapter 14

Reactions of (+) and W should be investigated further Table XIV shows results obtained using the four generally available antisera

TABLE XIV

Rh TESTING WITH FOUR ANTISERA

Anti C	*Anti D*	*Anti E*	*Anti c*	*Commonest genotype*
+	+	−	+	CDe/cde
+	+	−	−	CDe/CDe
−	−	−	+	cde/cde
−	+	+	+	cDE/cde or cDE/cDE Requires testing with anti-e or dosage test with anti E
+	+	+	+	CDe/cDE
−	+	−	+	cDe/cde
−	−	+	+	cdE/cde[1]
+	−	−	+	Cde/cde[1]

[1] In view of the frequency of Du in these genotypes they should be tested with a battery of anti D sera

IDENTIFICATION OF RHESUS ANTIBODIES

As a general rule antibodies giving strong reactions in albumin are rhesus antibodies To identify the antibodies it is necessary to

test the sera against a panel of group O cells selected to include as many antigens as possible Antibodies of other groups may also be present in the sera, genotyping of donor and recipient or parents being an invaluable lead in this investigation The tests should be done in saline and albumin at 37° C and also 16° C since very occasionally rhesus antibodies are found with this thermal optimum

REMOVAL OF UNWANTED AGGLUTININS

It is not always possible to remove rhesus antibodies from mixtures but sometimes success is obtained by absorbing the serum with an equal quantity of packed cells of the appropriate group at 37° C for 2 hours

The antibodies identified by a panel of cells are shown in Table XV

TABLE XV

IDENTIFICATION OF THE Rh ANTIBODIES

	rr *cde/cde*	R_1r *CDe/cde*	R_2r *cDE/cde*	R_0r *cDe/cde*	*R r* *cdF/cde*	*R r* *Cde/cde*	R_1R_1 *CDe/CDe*	R_2R_2 *cDF/cDE*
Anti D	−	+	+	+	−	−	+	+
Anti D C	−	+	+	+	−	+	+	+
Anti D E	−	+	+	+	+	−	+	+
Anti E	−	−	+	−	+	−	−	+
Anti-c	+	+	+	+	+	+	−	+
Anti C	−	+	−	−	−	+	+	−

TITRATION SCORES

Where a suspected serum contains another antibody in addition to one in the rhesus system it is tested by titration with those cells with *which it reacts and some with which it does not react as con-*trols The titrations are scored by applying a numerical value to the agglutination in the manner described in Chapter 15

Inspection of the titration scores will reveal a higher score for a double dose of reacting antigens and sometimes a still higher score for a single dose of each of two reacting antigens for example

Cells	*Score*	*Reacting antigens*
CDe/cde kk	20	c
cde/cde kk	26	cc
CDe/cde Kk	30	cK

Such a serum seems to contain anti c and anti K

The same scoring system is used for dosage tests as indicated in the first two lines of the example, the homozygote producing a higher score than the heterozygote

A RARE RHESUS ANTIGEN

A rare antigen in white people was described by Rosenfeld in 1953 and termed V Its precise place in the Rh system is not known but it is associated with cde and cDe Negro bloods tested gave positive reactions in 50 per cent of cases so that it is possible that transfusion of Negro blood to white people and mixed matings may cause immunization against the antigen

TRANSFUSION IMMUNIZATION

Individuals vary enormously in their ability to produce rhesus antibodies In the author s experience a D negative woman received 70 pints of D positive blood over a period of 5 years before developing antibody As an old woman suffering from a chronic bleeding condition it was considered wasteful to give D negative blood but in such cases a low titre antibody could by bad technique be overlooked The patient might take the blood without untoward effects but would receive no lasting benefit since the cells would disappear rapidly from the circulation Inapparent haemolysis of this sort is shown by a failure of the haemoglobin level to rise in the absence of further bleeding

Since the sensitization produced by one dose of rhesus antigens persists for many years and perhaps for life it is essential especially in females under menopausal age that the correct rhesus blood group is given

HAEMOLYTIC DISEASE OF THE NEWBORN

It is convenient at this stage to discuss haemolytic disease of the newborn Sensitization of the mother by an incompatible foetus results in the production of antibodies which circulate in the growing infant and may cause death *in utero* or a haemolytic anaemia of greater or lesser severity in the infant after birth The haemolytic triad termed erythroblastosis foetalis consists of (*a*) hydrops foetalis in which the oedematous foetus is born dead at or about full term (*b*) icterus gravis neonatorum in which the infant born apparently healthy becomes jaundiced within a few hours and dies quickly of kernicterus (poisoning of the basal ganglion of the brain by the products of haemolysis) or after a period of 7–21 days of profound anaemia (*c*) haemolytic anaemia of the newborn in which the child may be only mildly affected having what appears to be a physio

logical jaundice which takes some time to clear up, and remaining pale for some months

The manner in which sensitization occurs is not certain but it would appear that soluble antigenic material or actual tissue fragments are the agent rather than red cells Such antigenic material passes from the placenta into the maternal circulation causing the primary response Subsequent pregnancies are affected, the first one escaping unless there has been prior sensitization by transfusion The inheritance is shown in Fig 41 and it will be noted that every

(A)

♀ \ ♂	cDe	cDe
cde	cDe/cde	cDe/cde
cde	cDe/cde	cDe/cde

(B)

♀ \ ♂	cDe	cde
cde	cDe/cde	cde/cde
cde	cDe/cde	cde/cde

Fig 41 —(A) Mating of homozygotes (B) mating of heterozygous father with homozygous mother

child of the homozygous father is incompatible but only 50 per cent of possible progeny of the heterozygous father are incompatible It is possible in such cases to advise the parents about further children simply by genotyping

Erythroblastosis is not a constant feature of the disorder and may occur in infants born of diabetic mothers, in congenital heart disease and even in normal infants but severe cases may have as many as 200 000 erythroblasts per c mm (Fig 42) In any case more than 20 erythroblasts per 100 white cells seen in a stained film is a bad sign There may be 50–60 per cent of reticulocytes and polychromasia macrocytosis and other signs of blood regeneration present in the blood film Even the apparently healthy born infant who is going to be affected may have some anaemia resulting from red-cell destruction *in utero*, although 50 per cent of cases have a cord haemoglobin of 14 5 g per cent or more Kernicterus is far more likely when the cord haemoglobin is between 10 and 15 8 g



FIG 42—Peripheral blood in haemolytic disease of the newborn showing the erythroblastaemia responsible for the popular and misleading term erythroblastosis foetalis

per cent than when it is over this latter figure Mortality increases as the bilirubin level goes above 2 mg per cent but since the bilirubin cycle is not well established in infants the level is no measure of the haemolytic process although the more anaemic the infant the higher the bilirubin usually

Haemolytic disease of the newborn due to rhesus incompatibility occurs in 1-200 of all pregnancies and 95 per cent of cases are due to anti D ABO incompatibilities result in haemolytic disease in 1-2 000 to 3 000 of pregnancies and is usually mild in character Other blood groups are also involved and are discussed in the appropriate chapters

It seems clear that only the incomplete antibody is capable of passing the placental barrier hence the relative infrequency of ABO incompatible pregnancies Immune anti A and anti B do pass the placenta as do immune anti M and anti N The very infrequent incompatibilities due to MN antigens are due to the lack of potency of these antigens in man When the maternal serum contains predominantly incomplete antibody it passes the placenta readily but saline rhesus antibodies do not pass it at all It follows from this that the Coombs test on the infant s cells is the surest method of

detecting whether the child is going to be affected This may be very weak in cases of heterospecific pregnancies due to the ABO system but the lytic property of the maternal serum can be utilized to detect such cases The fresh maternal serum incubated with the infant s cells failing to lyse makes the diagnosis of haemolytic disease due to the ABO group unlikely

Jaundice in the newborn infant is not necessarily due to haemo lytic disease Congenital obliteration of the common bile duct, hepatitis and even hereditary spherocytosis can cause jaundice, but usually later than 48 hours after birth and the Coombs test is negative in these conditions

Treatment of the infant affected by haemolytic disease depends upon the severity of the condition assessed on the haemoglobin value and serum bilirubin level, but usually involves replacement trans fusion with cells which will not be affected by the antibody The process which is carried out via the umbilical vein also removes some of the free antibody Since the ABO agglutinins are not present at this early stage in cases of rhesus incompatibility blood compatible with the mother should be used for transfusion It may be necessary for transfusion to be repeated at intervals

ANTENATAL TESTING

In the normal course of events the ABO and D groups of pregnant women should be determined early in pregnancy For practical purposes only those women who are D negative are considered further Previous history of pregnancies or transfusion is very important and since sensitization may take place early in pregnancy abortions must count as previous pregnancies The blood must be examined for antibodies at the earliest opportunity and certainly at 28 weeks and again at 36 weeks Many cases only develop anti bodies late in pregnancy and the true titre is not seen until after the puerperium Weak antibodies detectable only by papain techniques and very weak Coombs test may be just as dangerous to the foetus as stronger antibodies hence every available method should be used for their detection

TECHNIQUE

The following are details of techniques relevant to data discussed in the first part of this chapter

PROVISION OF ANTISERA

(1) Collect blood from the donor into a dry sterile bottle and allow to clot at room temperature

(2) Store the bottle overnight at 4 C with the clot in contact with the serum to remove non specific cold agglutinins and next morning remove the serum in the cold
(3) Should the serum show any tendency to haemolyse the cells against which it is to be used inactivate it at 56 C for 30 minutes to destroy complement
(4) Absorb the serum at 4° C for 6–8 hours mixing every 2 hours with an equal volume of the appropriate red cells [1]
(5) Centrifuge the mixture to pack the cells and carefully remove the serum
(6) Test the serum at 20 C for 2 hours against 2 per cent suspension of A_1 and B cells of appropriate rhesus groups to ensure complete absorption and repeat absorption if necessary
(7) Finally dispense the serum in small amounts and store at − 20 C

Note Serum stored for months in small amounts tends to deteriorate faster than that stored in bulk It is suggested that sera are dispensed in small amounts only sufficient for a month s work

RHESUS GROUPING (D ANTIGEN) — SCREENING METHODS

Murray slide method saline anti D

(1) Wash cells twice with normal physiological saline and make up to 25 per cent suspension
(2) Place 1 drop of anti D on one end of platform of slide and 1 drop of cell suspension at other end of platform
(3) Mix by tilting slide and breathing on it to make drops run more easily
(4) Incubate 30 minutes at 37 C in moist chamber made of Perspex box with layer of moist lint at bottom
(5) Read macroscopically and microscopically

Murray slide method incomplete anti D

Exactly the same technique except that 1 drop of 20 per cent ox albumin is also added to mixture

Chown capillary method saline anti D

(1) Wash cells twice in normal physiological saline and make up to 25 per cent suspension
(2) Dip end of capillary tube (bore 0 4 mm critical) into saline anti D and allow serum to run halfway up the tube
(3) With finger over end of tube to prevent bubble of air at interface of two fluids dip end in cell suspension and allow to run up tube

[1] R d cells f r absorption re tak to ACD not more than 4 d y l r th t me of se and should be group A and B or A B a d hould la k th i gen to the ant body to be left in the serum

(4) Invert tube and stick in Plasticine at angle of 45°
(5) Incubate at 37° C
(6) Read after 5–15 minutes Positives show aggregate of cells along length of capillary negatives as smooth unbroken pink line

Note Serum must be of sufficient titre as to withstand threefold dilution with saline prior to use Neat sera or incomplete sera are not suitable

RHESUS GROUPING (D ANTIGEN)—TUBE METHODS

(1) *Wash cells twice with normal physiological saline and make up to* 2–5 per cent suspension
(2) To precipitin tubes add 1 drop of saline anti D and to each tube add 1 drop of each patient s cells
(3) Mix by flicking with the finger and cap tubes
(4) Incubate at 37 C for 2 hours and examine macroscopically and microscopically

Note Controls of O CDe/cde +
O Cde/cde −
A_1B D negative

Absorption control should be set up each time and where possible routine D typing should be done with more than one anti D serum to *pick up* D^u *antigen*

ALBUMIN REPLACEMENT TECHNIQUE

This technique can be adapted for use with incomplete anti D by using stages 1 2 and 3 and incubating for 1½ hours only At the end of this time carefully remove the supernatant serum and replace with an equal amount of 20 per cent ox albumin without disturbing the sedimented cells Then incubate for a further half hour and examine *macroscopically and microscopically*

Note The use of albumin creates an apparent haemolysed suspension but this is due to refractive indices and is not haemolysis at all

ALBUMIN ADDITION TECHNIQUE

Some sera are very suitable for this technique which avoids the laborious pipetting of the supernatant Instead of removing the serum an equal amount of albumin is carefully added to the tubes and the *tests reincubated for a further 30 minutes* Read in the usual manner

EMERGENCY RHESUS GROUPING

Tube centrifuge technique

(1) Wash the cells twice in normal physiological saline and make up to 25 per cent suspension in saline or in albumin 20 per cent

(2) Add 1 drop of saline anti D or albumin anti D to the tubes and 1 volume of cells in saline or albumin
(3) Incubate the tubes at 37 C for 15 minutes
(4) Centrifuge the tubes for 2-3 minutes at 2 000 r p m
(5) Read microscopically taking care not to mistake packed cells for agglutination

Note Controls are even more essential with this method

THE SANDWICH TECHNIQUE (STRATTON)

(1) Place 1 volume of anti D serum 1 volume of 30 per cent albumin and 1 volume of once washed packed cells on a microscope slide
(2) Mix the reagents and place a second slide on top wiping away any excess fluid from the edges
(3) Incubate at 37° C for 10 minutes and read microscopically

Pressing the slides with a finger allows the clumps to move ensuring that they are really agglutinated This technique works well with sera which are not potent enough to use in the tube or other techniques

D^u TYPING

(1) Wash the cells twice with saline and resuspend to 50 per cent and 2 per cent in saline
(2) Test the 2 per cent suspension with a minimum of five strong saline anti D sera Should some of the sera react and others not the cells are classed as High Grade D
(3) Expose the 50 per cent suspension to several powerful incomplete anti D sera for 2 hours at 37 C
(4) Perform Coombs test in treated and untreated cells Positive reactions with some or all the albumin antibody treated cells classify the cells as Low Grade D^u if they have not reacted with the saline antibodies The High Grade cells usually react with albumin antibodies also The untreated cells are tested by direct Coombs test to exclude false positive due to cold incomplete auto agglutinins

RHESUS GENOTYPING

(1) Wash the unknown cells twice in saline and resuspend to make a 2-5 per cent suspension
(2) Deliver 0 008 ml volumes of the appropriate sera to the bottom of precipitin tubes and add equal volumes of the cells
(3) Flick the tubes to mix incubate 2 hours at 37 C and if saline anti sera have been used read directly If albumin antisera have been used the replacement technique is recommended

The tests are read by transferring the deposited cells to a slide and read microscopically

IDENTIFICATION AND TITRATION OF ANTIBODIES WITHIN THE Rh SYSTEM

(1) Titrate the serum in saline and albumin against a panel of group O cells to include as many antigens as possible
(2) Add 1 volume 2 per cent suspension of cells to each tube and incubate 2 hours at 37° C
(3) Read microscopically

PAPAIN TECHNIQUE OF LÖW

Reagent

(1) Grind 2 g of papain in a mortar with 100 ml of M/15 phosphate buffer *p*H 5 4 and filter
(2) Add 10 ml of 0 5 M cysteine and dilute with the buffer to 200 ml
(3) Incubate 1 hour at 37° C

The incubated enzyme can be stored at − 20 C for many months

Method

(1) Mix 3 volumes of enzyme solution with 1 volume of serum
(2) Place 1 volume of mixture in a tube with 1 volume of 3 per cent suspension of appropriate red cells mix and incubate at 37 C for 2 hours
(3) Read macroscopically and microscopically

The technique can be used for rhesus typing with weak antisera or for the detection of weak antibodies

HAEMOLYSIN TECHNIQUE FOR ABO HAEMOLYTIC DISEASE

(1) To 1 volume of fresh maternal serum add 1 volume of once washed infant s red cells 2–5 per cent suspension
(2) To another tube add 1 volume of fresh maternal serum and once washed adult cells of the same ABO group as the infant 2–5 per cent suspension
(3) Incubate both tubes at 37 C for 2 hours Failure to lyse the infant s cells makes diagnosis of haemolytic disease due to the ABO group unlikely Failure to lyse the adult cells excludes haemolytic disease due to ABO incompatibility

CHAPTER 18

OTHER BLOOD GROUPS

THE LEWIS BLOOD GROUP SYSTEM

HISTORY

THE LEWIS blood group system was discovered by Mourant in 1946 using a naturally occurring antibody which reacted better at room *temperature than at 37 C The antibody differentiated those tested* into Lewis positive and Lewis negative but peculiarities of the group soon became obvious Andreson noted that Lewis positive children were produced in matings of Lewis negatives, and that the frequency of Lewis positive was much higher in children than in adults To explain these observations he suggested that in adults the group was behaving as a recessive characteristic that is not expressing itself in single dose while in the infant it was behaving as a dominant It follows that in the adult the Lewis positive individual is homozygous for the characteristic

A second peculiarity of the group described by Grubb in 1948 was its association with the secretion of ABH substances, Lewis positive individuals being non secretors

Another antibody defining a second Lewis antigen was described by Andreson in 1948 The reactions with most sera were almost the opposite of the original serum so the two antigens were termed Lewis (a +) and Lewis (b +) A specific notation for the system was agreed upon in 1949 and is shown in Table XVI

THE ANTIGENS

The Lewis antigens have been termed plasma or serum antigens This stems from the observation by Sneath and Sneath (1955) that when Le (a + b −) cells were incubated in Le (a − b +) plasma for 24 hours they reacted as Le (a + b +) On the other hand Le (a + b −) or Le (a − b +) cells incubated in Le (a − b −) plasma donated their antigens to the plasma Lewis antigens have always been regarded as tender in that repeated washings in saline remove the antigens The antigens are not very potent and in infants are weakly developed cord blood grouping as Le (a − b −) It is reported that during pregnancy the Lewis phenotype may alter

THE ANTIBODIES

The original Lewis antiserum, anti Le was naturally occurring and most antisera since described are of this type They react in saline suspension at room temperature producing a type of agglutination aptly described as stringy Potent Lewis antisera cause haemolysis of the appropriate cells at 37 C the effect being increased if the cells are previously treated with enzymes such as trypsin In complete Lewis antisera can sensitize red cells without agglutination but with some sera a positive anti globulin reaction cannot be obtained unless the sera are fresh The sensitizing agent is often not a γ globulin so that dilutions of Coombs reagent other than those used for Rh work may be necessary

TABLE XVI

THE LEWIS BLOOD GROUP SYSTEM

Antibodies	Anti Le^a Anti Le^b
Phenotypes	Le (a + b −) Le (a − b +) Le (a − b −) Le (a + b +)[1]
Genes or antigens	Le Le^b
Genotypes	Le Le Le Le^b Le^b Le^b

[1] Most cells of Group O and A_2 appear to belong to phenotype Le (a + b +) the characteristic A interfering with the expression of Le in heterozygotes

Anti Le^b is a less well understood antibody Many sera give reactions antithetical to anti Le but only if the tested cells are of Group O or A_2 Cells of Group A are either not agglutinated or only weakly affected This is said to be due to epistasis a genetical phenomenon in which the characteristic A interferes with the development of Le^b

Sera containing a mixture of the two Lewis antibodies are sometimes found and in such cases will react with the cells of most people In this case they have to be differentiated from anti H and anti O

THE LEWIS GROUP AND SECRETION

About 30 per cent of the English population are non secretors of ABH substances and these are all of phenotype Le (a +) With few

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THE KELL BLOOD GROUP SYSTEM

HISTORY

The original antibody which recognized the Kell antigen was described by Coombs, Mourant and Race in 1946 The antibody, which was of the incomplete type, was obtained from the serum of a woman who had been delivered of an infant affected by haemolytic disease The antigen K which was defined by the antibody is possessed by only 9 per cent of the English population, who are termed Kell positive With the discovery of the antibody Cellano by Levine and his associates in 1949 the inheritance of the group by means of two allelomorphic genes without dominance was proved and its independence from other blood group systems also shown

THE ANTIGENS

The antigens K and k are both potent and it has been suggested that they are tender in the same manner as the Lewis antigens Since the phenotype k— has a frequency of 91 per cent it is essential that individuals suffering from disorders where antibodies are easily produced should be Kell typed and blood donated accordingly Haemolytic anaemias particularly are liable to form anti K

THE ANTIBODIES

No naturally occurring Kell antibody has yet been described, all examples being derived from transfusion reactions or haemolytic disease Most of the antisera are incomplete behaving as crypt agglutinoids, but saline antibodies have also been described The antisera possess dosage properties although not well marked but since anti k can distinguish between the homozygote KK and the heterozygote Kk it is not important Anti K is often a serological contaminant of anti D sera, hence D negative K positive cells must be used for its demonstration

CLINICAL EFFECTS

Anti Kell can cause severe haemolytic transfusion reactions and haemolytic disease of the newborn Anti Cellano has caused mild haemolytic disease

THE DUFFY BLOOD GROUP SYSTEM

HISTORY

The Duffy blood group system takes its name from the patient who developed an immune antibody in response to transfusion

exceptions the phenotype Le (a —) are secretors of ABH substance Strangely however non secretors of ABH substance do secrete Lewis substance and the saliva of the phenotype Le (a + b —) may inhibit some anti Le^b sera

CLINICAL EFFECTS

Transfusion reactions due to Lewis antibodies are rare but have been described Their rarity may be due to the general weakness of the antibodies or lack of potency of the antigens Haemolytic disease due to the antibody does not occur even though the in complete antibody may cross the placental barrier since the anti gens may take several weeks to develop after birth

THE LUTHERAN BLOOD GROUP SYSTEM

HISTORY

The Lutheran blood group system was discovered by Callender and Race in 1946 using an antibody found in the serum of a patient who had multiple transfusions The patient suffered from lupus erythematosus a condition in which antibodies are readily produced and the Lutheran antibody was formed after the transfusion of blood from a donor of that name The antiserum agglutinated the red cells of 8 per cent of the English population tested and these were termed Lutheran positive The antigen was shown to be inherited as a Mendelian dominant and to be independent of other blood group systems A notation system similar to the Lewis system is used so that the antibody becomes anti Lu and phenotypes Lu (a +) and Lu (a —) The gene Lu^1 was only recognized as the absence of Lu until 1956 when anti Lu^b was described by Cut bush and Chanarin

THE ANTIGENS

The Lutheran antigens have not been described as causing either transfusion reaction or haemolytic disease From deliberate im munization experiments it would appear that the antigens are not particularly potent

THE ANTIBODIES

The Lutheran antibodies react in saline at room temperature the type of agglutination being characteristic—consisting of fairly large compact clumps in a sea of unagglutinated cells Cells can be sensitized to react with Coombs reagent

had been delivered of an infant suffering from haemolytic disease The group was shown to be inherited by two allelomorphic genes, the other antigen being identified by a serum discovered in 1953 by Plaut Ikin Mourant Sanger and Race What has become the accepted notation is followed the antigens being Jk and Jk^b and antibodies anti Jk^a and anti Jk^b The phenotype Jk (a − b −) has been described but is rare in white people Jk (a + b −) has a frequency of 25 per cent in England, Jk (a + b +) 50 per cent and Jk (a − b +) 25 per cent

THE ANTIGENS

The Kidd antigens seem to be well developed in infants and potency is variable

THE ANTIBODIES

It is necessary to use the anti globulin technique to demonstrate the antibodies and some sera will only react if the cells have been enzyme treated When Kidd typing this technique is recommended

CLINICAL EFFECTS

It seems possible that transfusion reactions can occur as a result of immunization with the Kidd antigens and haemolytic disease associated with anti Jk and other antibodies has been reported A few cases of haemolytic disease due entirely to anti Jk^a appear in the literature

LOW INCIDENCE BLOOD GROUP ANTIGENS

PRIVATE FAMILY ANTIGENS

Several blood group antigens have been described apparently confined to particular families The antibodies are rarities because the chances of sensitization by a single blood donation are small and the possibility of a second dose quite remote The antigens Levay Jobbins Mi Ven Ca Vw Be, Wr^a By^a and Rm are all private antigens but it is possible that such an antigen although rare in one race may be more common in another This is well illustrated by the Diego blood group system which was first classified as a family antigen in a Venezuelan of Caribe Indian ancestry Investigations have since shown that the antigen is not at all uncommon in South American Indians although it is a distinct rarity in white people The antibody defining the antigen Di^a is termed anti Di^a the notation following that of Duffy and similar groups although anti Di^b

The antibody described by Cutbush, Mollison and Parkin in 1950 behaved as a cryptoagglutinoid—being detected by the anti globulin reaction An antibody giving antithetical reactions was described by Ikin and his colleagues in 1951 It was shown that the antigens were inherited by two allelomorphic genes without dominance and a form of notation adopted like the Lewis and Lutheran systems

THE ANTIGENS

The antigens appear to develop early in life and have a racial distribution Quantitative differences in the antigens have been demonstrated In the English population Fy^a has a frequency of 66 per cent The antigens are potent but individuals vary greatly in their ability to react to the stimulus

TABLE XVII

THE DUFFY BLOOD GROUP SYSTEM

Antibodies	Anti Fy^a Anti Fy^b
Phenotypes	Fy (a + b −) Fy (a + b +) Fy (a − b +) Fy (a − b −)[1]
Genes or antigens	Fy^a Fy^b
Genotypes	Fy Fy Fy^a Fy^b Fy^b Fy^b

[1] Described in Negroes 68 per cent

THE ANTIBODIES

The original anti Fy was an incomplete antibody reacting only by the indirect Coombs test However in saline antibodies have since been described and dosage properties demonstrated Anti Fy^b is a much rarer antibody but both antibodies have been found in association with transfusion reactions and haemolytic disease

THE KIDD BLOOD GROUP SYSTEM

HISTORY

The Kidd blood group system was discovered by Allen Diamond and Niedziela in 1951, using a serum obtained from a woman who

(2) Incubate 2 hours at 37 C
(3) Wash three times with saline and test with anti globulin serum

N B Include known controls Sensitization time varies with anti serum and must be found by trial and error

DUFFY TYPING USING ANTI Fy^a SERUM

(1) Using the same technique as for Kell typing incubate for the appropriate time for the batch of serum at 37 C
(2) Perform Coombs test on sensitized cells

N B Include known controls Test negatives with another batch of serum

KIDD TYPING USING ANTI Jk^a SERUM

(1) Trypsinize unknown cells and known Jk positive and negative cells
(2) Incubate with anti Jk serum for 30–45 minutes in proportion of 2 volumes of antiserum to one half volume of washed packed trypsinized cells
(3) Wash three times and perform Coombs test on cells

has not been described The antibodies of the private antigens react mainly by the anti globulin technique but saline antibodies have also been described

HIGH INCIDENCE BLOOD GROUP ANTIGENS

PUBLIC ANTIGENS

The converse of a private antigen is one which is very widely distributed Such antigens are termed public antigens and the rare recipient who lacks the antigen has a very good chance of becoming immunized The antigens H Tj Vel and Yt are regarded as public antigens the antibodies being anti Tj, anti Vel and anti Yt ordinarily active at 37 C and some samples having haemolytic properties

TECHNIQUE

The following are details of the techniques appertaining to the blood group systems tabulated in the earlier part of this Chapter

LEWIS TYPING WITH ANTI LE SERUM

(1) Place equal volumes of antiserum and 2 per cent suspension of red cells in precipitin tubes
(2) Incubate 2 hours at room temperature and read microscopically

N B Wash cells three times with saline since although the cells are tender the antigens are also present in the serum and may cause inhibition of the testing serum

Use at least two different anti Le sera

Include known Le positive and Le negative cells as controls

DIFFERENTIATION OF MIXED ANTI LE + ANTI LE' FROM ANTI O OR ANTI H

Using standard technique for Lewis typing test the serum against several cells of group O Le (a — b —) If reactions are positive the serum is not anti Lewis mixture

LUTHERAN TYPING USING ANTI LU SERUM

Using the same technique as for Lewis typing incubate at the known optimum temperature (usually 18–20 C) for 2 hours

N B Include known controls Handle deposited cells gently

KELL TYPING USING ANTI K SERUM

(1) Mix 2 volumes of anti K serum with one half volume of washed packed fresh cells

system with elliptocytosis is proved but not enough markers are available for other characteristics to be labelled with certainty The ability to taste phenyl thio carbamide (PTC) in a dilution of 1 20 000 in tap water is a non blood group marker used in linkage studies The gene for tasting the compound, an unpleasant bitter material is called T and is dominant to its allele t for non tasting It is not a clear cut marker since there appears to be a sex difference but linkage with the blood group systems has not been demonstrated However with the nine major blood group systems it provides us with ten marker genes

THE DISTRIBUTION OF THE BLOOD GROUPS

Knowledge of the blood groups of different races and nationalities is of great value in ethnology Study of the frequencies has enabled conclusions to be drawn regarding migrations of populations in the past although some cases would seem to conflict with other evidence The ABO frequencies in Iceland Scotland and Northern Ireland (not English settlers) are very similar and two conclusions may be drawn from this fact Firstly it may be suggested that the Icelandic settlers originally came from Scotland or Ireland but history tells us that the first colonizers were Vikings The modern Scandinavian ABO frequencies may however result from dilution with an Asiatic phenotype in which case the original settlers may have been Scandinavian Evidence for the invasion of Europe by an Asiatic race is gleaned from a study of ABO and especially Rh frequencies To explain the high frequency of the d gene when there is constant selection against the heterozygote exerted by haemolytic disease, it is suggested that the present frequencies result from dilution of the predominantly Rh negative original Europeans with an Rh positive invader The high frequency of D among Negro and Chinese populations accords with this theory of an Asiatic invader As a further example of blood groups in ethnology it is surprising to find that the Polynesian tribes resemble South American peoples in their ABO frequencies whereas they are believed to have originated from S E Asia It may be that the use of blood groups in this instance may alter theories of migration in this part of the world

RED CELL SURVIVAL

The life span of normal red cells has been established by many methods the modern one of tagging with a radioisotope having already been described (*see* Chapter 13) However by transfusing compatible cells of different blood group to a normal individual

CHAPTER 19

USES OF BLOOD GROUPING

GENERAL

INTRODUCTION

ALTHOUGH the most obvious use of blood grouping is in blood transfusion to save life it is of great value in other directions Medico legal aspects have already been mentioned, both in respect of disputed paternity and identification of blood stains and to this must be added the investigation of haemolytic disorders by red-cell survival studies Less well known is the use of blood grouping in human genetics applied to ethnology and anthropology

MARKER GENES

Some characteristics can be shown to be inherited together so consistently as to leave no doubt that the genes are on the same chromosome Where such linkage can be established the genes are known as markers and the more marked genes there are available the easier it is to prove further linkage Much work has been done with the fruit fly Drosophila with the production of maps showing the actual order of genes on the chromosomes In humans the sex chromosomes are better known in this respect but the blood group genes are valuable as autosomal markers since they are not sex linked Unlike many physical characteristics whose inheritance is multigenic the blood group genes also possess the advantage of controlling single antigens Although crossing over disturbs linkage studies it can be used to determine the closeness of the linkage

LINKED CHARACTERISTICS

Most of the blood group systems segregate independently but it would seem that the Lewis and Lutheran systems are inherited by genes showing autosomal linkage Attempts to show linkage between blood group systems and other inherited characteristics include elliptocytosis Mediterranean anaemia sickle cell anaemia and acholuric jaundice as red cell defects but normal characteristics such as red hair have also been investigated Linkage of the Rh

CHAPTER 20

BLOOD TRANSFUSION

GENERAL

HISTORY

ALTHOUGH the original suggestion came from Andreas Libavius in Italy as early as 1615 there is no record of a successful attempt at blood transfusion until 1666 when Lower, having almost completely exsanguinated a small dog resuscitated the animal with blood from another dog A year later Denys in Paris transfused blood from a calf to a dog and later still reported the transfusion of a small amount of sheep blood to a human being Domestic animals were frequently used as donors after this, particularly in cases of post-partum haemorrhage but it was not until 1818 that Blundell reported a successful human blood transfusion having first shown that the blood of one species was harmful to another

The greatest difficulty in the early days of blood transfusion arose from clotting in the apparatus used At one time defibrinated blood was used but was eventually rejected mainly it would seem because its protagonists were also exponents of the use of animal donors Sodium phosphate oxalates peptone and hirudin were tested as anticoagulants but were found to be toxic or frankly poisonous Sodium citrate rejected as toxic by Lespinasse in 1908 was successfully used for the first time by Agote in 1914

The danger of transfusion within human beings was appreciated by Higginson in 1857 but the nature of the danger was only fully understood with the discovery of the blood groups by Landsteiner (1901), Jansky (1907) and Moss (1910)

ABO TRANSFUSION POSSIBILITIES

With the discovery of the ABO blood group system it became obvious that transfusion between the groups was limited by reason of the naturally occurring antibodies The possible donors and recipients within the system are shown in Fig 43 Unfortunately, Group O blood because of its apparent safety became known as that of a Universal Donor and was used for recipients of all ABO groups This practice, in addition to being a wasteful misuse, also caused

it is possible to determine the life span of the transfused erythrocytes by blood grouping methods In haemolytic anaemias the technique has been used to decide whether a red cell defect or a plasma factor is responsible for the haemolytic process Ashby s (1919) technique of indirect differential agglutination consisted of transfusing Group O blood to recipients of other groups The mixture of cells was then treated with the appropriate agglutinating serum leaving the donor cells free The direct differential agglutination technique aimed at the donor cells leaving the recipient s cells unagglutinated

It is essential that very potent antisera are used in this technique which can be extremely accurate within the limits of error of a red cell count The available sera limit the method to the ABO and MN groups but work with the Rh system has been described

TECHNIQUE

DIFFERENTIAL AGGLUTINATION (DACIE AND MOLLISON 1943)

The following describes differential agglutination technique

(1) Add 0 1 ml venous blood to 4 9 ml of 3 per cent sodium citrate and mix well making a dilution of 1 50
(2) To 1 volume of this suspension in a 75 × 10 mm tube add 1 volume of the appropriate potent agglutinating serum mix well and stopper with a well fitting rubber bung The dilution is now 1 100
(3) Allow to stand at room temperature 2 hours and then centrifuge at 1 500 r p m for 1 minute
(4) Shake vigorously so that the large agglutinate is broken into smaller fragments and allow to stand 1 minute
(5) Remove the upper three quarters of the suspension consisting of free cells and small clumps and centrifuge this mixture 1 minute at 1 500 r p m
(6) Well mix by the standard procedure of 50 inversions through an angle of 90–120 degrees at the rate of one per second
(7) Fill chamber from the upper layer of this suspension and after allowing the cells to settle count the free cells

N B Pre transfusion sample should show a blank of no more than 10 000 free cells per c mm when blood containing 5 mills /c mm. is agglutinated by this technique

The test is repeated at intervals post transfusion and the number of unagglutinated cells present reported either in absolute numbers or as a percentage of the number present 24 hours after transfusion

Extensive operations are possible with modern technique in anaesthesia and such procedures require cover ' with compatible blood The demands in some cases may be very large as in operations using a heart lung machine, or small when designed to counteract surgical shock rather than actual blood loss

Burns which involve plasma loss are best treated with the appro priate fluid but there may be indications for blood transfusion if burning is extensive and haemolysis results Loss of fluid by vomit ing diarrhoea or exudation into the bowel during obstruction does not require transfusion of blood

Blood is not a tonic and although some conditions may require treatment with blood or its products in the absence of actual blood loss there seems to be no valid reason for blood to be given to the inoperable cancer *in extremis* Blood is a dangerous substance and although it is a most valuable one should not be prescribed indis criminately

HAZARDS OF TRANSFUSION

Avoidance of transfusion hazards is properly the responsibility of the clinician and the dangers are mentioned here only briefly Local reactions such as haematomata thrombosis and sepsis at the site of transfusion can in great measure be avoided by the correct technique Circulatory overloading is a problem which can arise from a wrong assessment of the needs of the patient Citrate intoxication can theoretically ensue if a great amount of citrated blood is transfused very quickly and this has been reported in infants Interference with the coagulation mechanism is unlikely since the concentration of citrate necessary would probably cause death Potassium intoxication theoretically can arise from the use of stored blood since the potassium quickly diffuses out from the red cells but this is unlikely to occur unless the patient s blood potassium level is already raised and several pints of more than ten day-old blood transfused

Simple febrile and allergic reactions to transfusion are very com mon and are usually controlled by the administration of antipyretics and antihistamine drugs

Two other clinical considerations must be mentioned here Firstly the possibility of air embolism a condition which should never occur despite the ability of the body to withstand small amounts of air intravenously The positive pressure is usually produced by means of a Higginson syringe but a pump using pres sure outside the apparatus and literally rolling the blood forward is

many recipients to become sensitized to other blood group antigens It is to be regretted that the practice still survives in a modified form blood of Group O Rh negative being regarded as that of a universal donor This is even more wasteful because of the comparative rarity of the blood concerned and the dangers of sensitization still apply

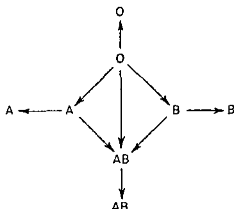


FIG 43—Transfusion possibilities within the ABO blood group system

Individuals of Group AB are still referred to as Universal Recipients but this is also a misnomer because of the possibility of sensitization to antigens outside the ABO system In addition to this danger inadequate grouping technique might wrongly label blood of Group A_2B as that of AB The transfusion of A_1 or A_1B blood to such an individual could be dangerous since the plasma of the recipient might contain a high titre alpha agglutinin

INDICATIONS FOR TRANSFUSION

Loss of blood from any cause is sufficient reason for transfusion providing the loss is great enough A loss of 500 ml from a healthy donor is rapidly made up without assistance but the trauma attending an accidental loss of the same amount could cause symptoms of severe shock which might be considered grounds for transfusion As the volume of blood loss increases above 500 ml so the shock becomes more severe and the necessity for transfusion more urgent A sudden loss of 2 500 ml—which in a ten stone man is equivalent to half the normal blood volume—will inevitably be fatal unless transfusion is initiated quickly

Simple anaemia is not an indication for transfusion unless treatment with haematinics fails The haemolytic and haemorrhagic anaemias require transfusion depending on the extent of the anaemia and the marrow activity Aplastic conditions require periodical transfusions since the marrow is incapable of producing blood cells

A curious transmitted disorder has been recorded in that allergies of the donor may appear temporarily in the recipient. Although this seems a short lived effect it is best not to use donors who suffer from allergic disorders.

SELECTION OF DONORS

Apart from considerations of disease already mentioned the donor is selected on his general health, haemoglobin level, ABO and Rh group and for special purposes his blood genotype for other antigens. The donor should not have been in contact with any infectious disease and should have a haemoglobin level of at least 12.4 g per cent. At the time of donation blood should be taken to check the blood group and for serological testing for syphilis. The serum of the donor must be checked for high titre naturally occurring antibodies since such donors can be dangerous. Immunization by foreign protein can cause a rise in titre of naturally occurring antibodies, *a point to be checked carefully in Service personnel* who receive inoculations against tetanus and typhoid.

RED CELL SURVIVAL

In spite of the fact that no obvious haemolysis is present in a bottle of stored blood, many of the red cells have lost their power to survive *in vivo* so that on transfusion they are rapidly removed from the circulation. One of the factors governing post transfusion survival of red cells is their content of organic phosphate and dextrose that provide energy for the synthesis of such compounds, particularly diphosphoglycerate and adenosine triphosphate. The ACD mixture used as an anticoagulant today is very satisfactory for 14 days storage of blood but the post transfusion survival of red cells diminishes progressively so that, at 4 weeks about 30 per cent are destroyed within 24 hours and the others survive for a much shorter period than fresh cells.

New anticoagulants are compared for red cell preservation by the Ashby (*see* Chapter 19) or similar technique. An ancillary technique used is osmotic fragility but cells apparently fragile in saline solutions often survive quite well on transfusion.

COLLECTION APPARATUS

The apparatus used for collection of blood from donors consists of a sterile bottle containing 120 ml of acid citrate dextrose (ACD) solution fitted with *a perforated aluminium cap and rubber liner.* Some bottles have a pilot bottle fixed by a metal bracket to the neck. The taking set itself consists of 18 inches of $\frac{3}{8}$ inch diameter rubber

preferable since bacteria may be introduced into the bottle by the Higginson syringe

Secondly, the rate of transfusion is arbitrarily fixed at 40 drops per minute but this can be markedly increased in case of need The blood may even be given intra arterially if necessary, and it must be rare that reactions occur due to blood being given too fast in cases of blood loss Blood given by this method must be under pressure Septic conditions and severe anaemias, however may require the blood to be given slowly since reactions may occur due to fast transfusion The aetiology of the reaction in many of these cases is obscure

Haemolytic reactions may be due to the adminisfration of incompatible or infected blood or to blood which has undergone haemolysis Clinically the picture is the same in all cases although properly only the first should be termed a haemolytic reaction The investigation of such reactions is discussed under the appropriate section

The transmission of disease via the transfusion needle is a very real danger Although it is unlikely that donors with bacteraemia would present themselves theoretically any such condition could be transmitted to the recipient The great dangers are syphilis malaria and homologous serum jaundice, but cases have been recorded of typhoid typhus smallpox and measles following transfusion of blood donated during the incubation period

The transmission of syphilis is avoided by testing the donor s serum and storage of the donated blood at + 4° C for a period of 6 hours Testing of the donor s serum by Kahn or a similar technique is not always a certain method of eliminating the undesirable donor since cases of primary syphilis are often sero negative The storage of blood however seems to kill the spirochaete

Opinions differ as to the viability of the malaria parasite in stored blood and by far the safest course is to reject as donors not only those who have suffered from the disease but also those who have lived in a malarious area without showing signs of infection

Homologous serum jaundice (virus hepatitis) is a disease which ranges in severity from a mild inflammatory liver disorder to actual liver necrosis It would appear that the virulence of the virus is often enhanced by what is in effect animal passage that is direct transfusion of the virus during the incubation period from an apparently healthy donor to the recipient Under these circumstances a certain incidence of the disease is to be expected among transfused patients but this can be decreased by questioning the donor Any donor giving a history of contact with a jaundiced individual must be rejected if the contact was within a period of 3 months

Needles after soaking are rinsed through with a strong jet, the inside of the butt cleaned with an applicator and cotton wool and the lumen cleared with a stiff wire They are then washed again dried and resharpened Finally they are soaked overnight in trichlorethylene to remove oil remaining from the sharpening drained and polished with a cloth and rinsed through several times with methylated spirit. Adapters and screw clips are soaked overnight cleaned, rinsed in distilled water and dried

The sets are assembled, taking sets packed in tin boxes in dozens and sterilized at 20 lb for 30 minutes The giving sets are assembled wrapped in Cellophane and sterilized individually in tin boxes at the same pressure and for the same time

TECHNIQUE

The following techniques are those applicable to blood transfusion as detailed in the first part of this Chapter

PREPARATION OF KAHN ANTIGEN

(1) Obtain fat free beef heart muscle and mince finely
(2) Spread the material on a tray to dry grind to a powder as it dries
(3) Extract with 400 ml ether for each 100 g dried heart powder
(4) Repeat extraction with 300 ml ether three times removing ether by suction filtration and using a fresh filter paper each time
(5) Dry the powder until no smell of ether remains
(6) Add 5 ml 90 per cent ethanol for each gramme of powder and shake for 10 minutes
(7) Store at room temperature in the dark for 3 days without shaking
(8) Shake for 10 minutes and filter
(9) Add cholesterol to make a final 1 per cent aiding solution by gentle heat
(10) Cool and refilter
(11) Store in a stoppered bottle in the dark at room temperature

STANDARDIZATION OF KAHN ANTIGEN

(1) Prepare six mixtures of antigen and saline by pouring the saline rapidly into the antigen and pouring back and forth twelve times Use 0 9 1 1 1 3 1 5 1 7 and 1 9 ml of saline to 1 ml of antigen
(2) After 10 minutes standing place 0 0125 0 025 and 0 05 ml of each antigen mixture in the appropriate tubes and add to all tubes 0 15 ml of saline Deliver the antigen to the bottom of the tubes
(3) Shake to mix then in shaking machine for 3 minutes
(4) Add 1 ml of saline to each tube with 0 05 ml antigen and 0 5 ml saline to the others
(5) Shake to mix and examine with a hand lens for undispersed flocculation

tubing with an olive mount needle at each end The taking needle *is 14 S G, sharpened to a medium bevel and having cutting edges* It is fitted with a stilette and mounted in a glass tube The other needle is of the same size but is blunt and again is protected with a glass tube Although not all taking sets are so equipped it is useful to have a glass 'window let into the tubing at the taking end A third needle is set in rubber tubing attached to a glass air filter consisting of a plug of cotton wool This is the standard M R C taking set

THE GIVING SET

The standard giving set consists of a 16 S G needle protected with a stilette and enclosed in a glass tube The needle is set in a short length of rubber tubing at the other end of which is a metal male adapter The female adapter is fitted to 7 inches of ⅜ inch diameter rubber tubing which joins the drip chamber The drip chamber itself is attached by 40 inches of rubber tubing to a short length of glass tubing set in a rubber bung Also set in the rubber bung is a long piece of glass tubing fitted with a small rubber collar The gas mantle filter ties in place above this collar and around a groove in the rubber bung Both this set and the taking apparatus will soon be superseded by plastic disposable sets having the added advantage of lightness

CLEANING AND STERILIZATION OF APPARATUS

After use blood bottles are washed, preferably on a machine of the type which cleans milk bottles They are cleared with strong jets of warm water inside and out automatically brushed inside with the assistance of a detergent and then several jets of warm water Finally they are treated with steam under pressure and dried upside down The caps are soaked in tap water overnight scrubbed to remove dried blood and boiled in distilled water for 10 minutes Finally they are rinsed in distilled water and dried in an oven The rubber liners are discarded since they have been punctured during use

The rubber and glass components of giving and taking sets after use are soaked in tap water overnight the gas mantle filters being discarded All glass parts are then rinsed in tap water and boiled for 10 minutes in distilled water and soap powder They are then thoroughly rinsed in tap water rinsed in distilled water and dried in a hot air oven The rubber parts are treated similarly except that no soap powder is used They must however, be scrubbed to remove dried blood tubing being cleaned with a burette brush

The titre to use is indicated by the least amount of saline with complete dispersal of the floc

KAHN TEST PROPER

(1) Inactivate test sera for 30 minutes at 56° C
(2) Prepare antigen according to titre and allow to stand 10 minutes
(3) Use three tubes for each test In the first place 0 05 ml in the second 0·025 and in the third 0 0125 mL of antigen delivering to the bottom of the tubes
(4) Add 0 15 ml of serum to each of the three tubes
(5) Shake for 3 minutes in the mechanical shaker
(6) Add 1 0 ml of saline to that tube containing 0 05 ml of antigen and 0 5 ml of saline to the other tubes
(7) Shake to mix and read with a hand lens

Reading results

Allot a number to each tube according to the degree of flocculation

Large flocs complete clearing of solution	++++ (4)
Large flocs fluid opalescent	+++ (3)
Flocs not readily seen by naked eye but easily with × 5 lens	++ (2)
Flocculation only visible with lens	+ (1)
No flocculation Uniform opalescent fluid	Negative (0)

The results may be reported as the mean of the three readings on the numbers separated as a series e g ++++ +++ ++ = +++ or 4 3 2 = 432 The latter method is preferable in that it can indicate a falling titre level titre or rising titre

PRICE PRECIPITATION REACTION (P P R)

Preparation of antigen

(1) Select fat free beef heart muscle and mince finely Grind with sand or broken glass
(2) Add 5 0 ml of absolute ethanol to each gramme of ground heart in a dark glass bottle and shake thoroughly
(3) Store at room temperature for 3–4 days shaking two or three times a day
(4) Filter contents into another glass stoppered bottle and keep at 4 C for 24 hours
(5) While still cold re filter into a dark glass stoppered bottle The antigen is stored at room temperature

STANDARDIZATION OF ANTIGEN

(1) Into four tubes place 1 0 ml of antigen add 0 4 0 6 0 8 and 1 0 ml of saline mixing saline rapidly into antigen pouring back and forth six times

(2) Allow the mixtures to stand 30 minutes, then centrifuge at 2 000 r p m for 10 minutes
(3) Discard the supernatants by inversion and allow to drain upside down on to filter paper for 5 minutes
(4) Dry the inside rims of the tubes and add 0 6 ml of saline mixing thoroughly with a pipette This antigen is stable for 4 days if kept at 4° C when not in use
(5) Set up five sets of three tubes and in one of each set put 0 11 ml of saline in another 0 11 ml of negative serum, and in the third 0 11 ml of moderately positive serum
(6) Using one set of three tubes to each antigen preparation place 0 022 ml of antigen in each tube
(7) Shake for 5 minutes
(8) Add 1 ml. of saline to each tube and read with a hand lens The titre for use is the least showing no residual floc in either the saline or negative serum tubes This is almost always 0 6 ml

P P R TEST PROPER

(1) Inactivate test sera for 30 minutes at 56 C
(2) Use two tubes for each test In the first place 0 055 ml of saline and 0 055 ml of serum and in the other 0 11 ml of serum Add 0 022 ml of antigen to each tube
(3) Shake the tubes for 5 minutes
(4) Add 1 0 ml of saline to each tube and read against a strip lamp or over a concave mirror

Reading results

Flocs visible to the naked eye fluid clear	+ (strong)
Flocs just visible to naked eye	+ (weak)
Flocs just visible with lens	± (trace)
Uniform opalescent fluid	Negative

The Price Precipitation Reaction is the method of choice because of the ease of reading results stability of antigen and greater specificity than the Kahn test

Rapid haemoglobin estimation

(1) From a height of 1 cm allow a drop of donor heparinized blood (or blood from a finger prick) to fall into copper sulphate solution S G 1052 (*see* Chapter 2)
(2) Observe action of copper proteinate sac for 15 seconds If drop sinks haemoglobin is over 12 4 g per cent which is equivalent to 85 per cent on the Haldane scale

PREPARATION OF A C D SOLUTION

Disodium citrate ($Na_2H(C_6H_5O_7)\ H_2O$)	2 g
Dextrose anhydrous	3 g
Distilled water pyrogen free	120 ml

(1) Filter first through filter paper and then through sintered glass
(2) Bottle and screw caps on lightly Cover with small brown paper bags
(3) Autoclave at 20 lb for 30 minutes and leave overnight in sterilizer
(4) Screw caps down tightly and secure paper bags with elastic bands

The solution shows slight evidence of caramelization This is not a disadvantage since it indicates that the solution has been sterilized Should the bottle show any sign of scaling it is discarded

TEST FOR PYROGENS (*see* Chapter 23)

The A C D solution must be tested for its pyrogenic activity (power of producing a rise in temperature) and a biological test is used for this

(1) Obtain three healthy rabbits of either sex each weighing not less than 1 5 kg and having a rectal temperature not exceeding 39 8 The animals should not have been used for previous tests in the preceding 3 weeks and not at all if its temperature rise in a preceding test was significant since exposure to pyrogens reduces the animal s response
(2) Inject the animals intravenously with 10 ml per kg body weight of 0 9 per cent pyrogen free sodium chloride 3 days before the test Animals showing undue rise in rectal temperature must not be used for the test

N B Food is withheld from the rabbits overnight and water withheld during the test

(3) Two hours before injections start taking rectal temperature at intervals of 20 minutes
(4) Inject intravenously 10 ml per kg body weight of A C D mixture
(5) Take rectal temperatures at 20 minute intervals for 3 hours after injection

The mean initial temperature of each rabbit is the mean of the temperatures recorded for the rabbit in the 40 minutes immediately preceding injection The maximum temperature of each rabbit is the highest temperature in the 3 hours after injection The difference between the mean initial temperature and the maximum temperature is its response If the summed response does not exceed 1 15 C the material is passed as pyrogen free but if the summed response exceeds 2 65 C it is discarded and investigations started to discover the cause

CHAPTER 21

BLOOD AND BLOOD PRODUCTS

GENERAL

FRESH BLOOD

USED in this context the term fresh blood implies modified blood which has been stored not more than 24 hours Such blood is often credited with properties denied the usual stored blood although in many cases there would appear to be little ground for this

The haemorrhagic diatheses are the conditions in which fresh blood or plasma is of the most value Stored blood is just as effective as fresh in controlling the haemorrhage of Christmas disease but anti haemophilic globulin disappears rapidly on storage It is necessary to decant the plasma rapidly from fresh blood and store it frozen solid to provide the cover necessary for operative procedures in haemophilia The plasma should be prepared not earlier than 24 hours before use Many fibrinogen preparations contain anti haemophilic globulin but in amounts too small to be of value Hypoprothrombinaemia is another indication for treatment with fresh blood and in infants the haemorrhagic tendency is also treated with vitamin K The depletion of Factor VII produced by the administration of dicoumarol may be rapidly made good by fresh blood transfusion although this factor also survives in stored blood However since the haemostatic defect in the prothrombin and allied deficiencies may be more than a simple one it would seem that fresh blood is the treatment of choice in haemorrhage associated with these conditions The bleeding which occurs in hypofibrino genaemia either due to severe liver disease or the acute defibrination syndrome following lung surgery or obstetric conditions is treated with whole blood plasma or injections of fibrinogen

PLATELET TRANSFUSION

The transfusion of whole platelets is indicated in those conditions in which platelet production is impaired but where platelet anti bodies are present it may be a waste of time and labour Platelets survive in citrate plasma but qualitative changes occur and it is preferable that the blood be collected into sequestrene (20 ml

4 5 per cent EDTA in 0 7 per cent NaCl per 500 ml blood) Either siliconed collecting apparatus is used or alternatively blood is collected into special plastic bags which can be centrifuged Using the plastic sets the platelet rich plasma is transferred to another bag by merely squeezing so that it is forced through an outlet tube Either the plasma is transfused or the platelets, packed by centri fuging and resuspended in saline are given alone

CONCENTRATED CELL SUSPENSION

The patient with a severe red cell anaemia does not need the plasma of modified blood and in such cases a concentrated cell suspension is used This is simply prepared using the apparatus (Fig 44) described near the end of this Chapter, whereby the cells of two bottles are mixed ensuring that some plasma-citrate is left to give a final packed cell volume of about 70 per cent Since there is always the possibility of infection in any process involving opening the bottles concentrated cell suspensions must be used within 12 hours

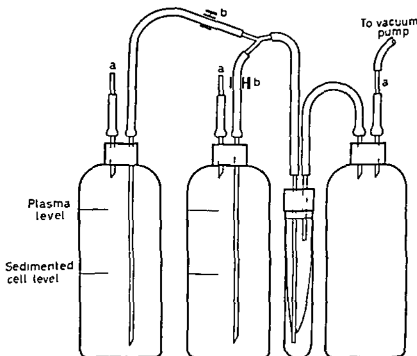


FIG 44 —Apparatus for the preparation of concentrated cell sus pension *a* = air filter *b* = screw clip *c* = nylon filter from standard giving set

PLASMA FREE TRANSFUSION

It is not possible without a great deal of trouble to produce a completely plasma free cell suspension, but in some conditions washed cells are necessary Sufferers from paroxysmal nocturnal haemoglobinuria may experience transfusion reactions unless well-washed cells are used and the rare plasma reactor also requires such a preparation Where it is desired to give blood free of isoagglutinins the cells must also be washed A refrigerated centrifuge able to take the M R C bottle is very useful for this process Once again aseptic conditions must be observed during the washing and the cells finally resuspended in sterile saline

EXCHANGE TRANSFUSION

In haemolytic disease treatment is aimed at removing the child s affected red cells and replacing with cells which will not be affected by the particular antibody concerned Efficient transfusion of 60 ml blood per lb of body-weight should leave not more than 500 000 per c mm of cells which are liable to be affected by the antibody Exchange transfusion at the same time removes a considerable amount of the free antibody from the plasma The cell content of the bottles is concentrated by removing 200 ml of plasma citrate from each bottle This avoids citrate toxicity and overloading with fluid Calcium gluconate is sometimes injected to counteract the citrate effect which of course is avoided completely if heparinized blood is used

The blood to be transfused should not contain the antigen corresponding to the antibody causing the disease and should as far as possible be of the same ABO group as the infant unless an ABO incompatibility is responsible for the condition The cross match should be performed against the maternal serum if ABO compatible since there may be little or no free antibody present in the child s plasma The antibody may be eluted from the cells of the infant, and the eluate (*see* Chapter 8) used in the cross match but there is no practical advantage in this technique

In a very small percentage of cases of haemolytic disease due to rhesus incompatibility it is not possible to use the maternal serum because of ABO incompatibility In such cases the ABO anti bodies of the maternal serum may be neutralized by the addition of secretor saliva or even a potent serum of the same specificity as the maternal serum used If these techniques or an eluate of the infant s cells are used the cross match can be further checked by using the child s serum

Exchange transfusion in haemolytic disease due to ABO incompatibility presents special problems Group O blood is used, of the same rhesus group as the infant, and the cells washed to remove iso-agglutinins To replace the protein the cells are re suspended in reconstituted pooled plasma or plasma of the same group as the recipient

The routes chosen for the administration of the blood depend on the clinician but in the first few hours of life the umbilical vein is usually the choice a catheter being passed into the vessel Alternatively the internal saphenous vein or scalp veins are used although both these routes are usually reserved for transfusions necessary after the first 48 hours Blood is withdrawn in 10–20 ml amounts discarded and an equal quantity of modified blood introduced by means of a syringe with a four way tap The process is repeated until the two bottles have been used or until the clinician is satisfied that sufficient has been done for the time being The technique aims at providing the infant with a haemoglobin value of 17 g per cent the concentration of transfused cells having a depressing effect on the marrow This is important because it allows fewer red cells to enter the circulation to be destroyed If the haemoglobin value can be maintained above 9 g per cent for 8 weeks, the antibody will have almost disappeared and the risk of further lysis diminished accordingly The most obvious danger of the syringe technique is air embolism which however is easily avoided by keeping the syringe piston uppermost and discarding bubbles as they are seen

CROSS MATCHING

Complete cross matching implies that the donor blood is tested against the recipient serum in such a way that all possible antibodies are included This means that the test is put up in saline and albumin at room temperature and 37 C and indirect Coombs test included Under these conditions the iso agglutinins and other lower temperature antibodies are detected in saline at room temperature and the albumin test at this temperature will detect cold incomplete antibodies including the rare incomplete Lewis antibody These of course are also detected by the indirect Coombs test The warm antibodies are detected in saline and albumin at 37 C Naturally occurring cold agglutinins that is active at 4 C are not important but if they are of wide thermal amplitude can cause trouble Such antibodies will be detected at room temperature and even at 37 C Enzyme methods are often used in conjunction with other compatibility tests Trypsinized

cells constitute the most sensitive indicator of weak rhesus anti bodies but papain techniques which fail to detect some Duffy and Kell antibodies are not recommended

Emergency cross matching presents difficulties but the albumin centrifuge technique is satisfactory especially if combined with Coombs' test Slide cross matching in emergency is dangerous, a tube test for as little as 30 minutes being more satisfactory Should the emergency be even greater, blood of Group O Rh negative is used without cross match on the responsibility of the clinician With plasma expanders available such emergencies should be rare

It is wise to include an auto agglutinin control, that is, the patient's serum against his own cells If agglutination occurs it will usually mean incompatibility in the tests against donor cells since such antibodies are often pan agglutinins

THE USE OF BLOOD PRODUCTS

The use of fibrinogen in the acute defibrination syndrome has already been mentioned but it is possible that it might be valuable in other conditions Plasma fibrinogen is decreased in the second week of virus infections and remains low for as long as 6 months It is very low in acute yellow atrophy and hepatic disturbances following severe burns, but plasma rather than fibrinogen is usually given in such cases

Variations in total serum proteins are not of clinical importance unless they are very large and also qualitative Chronic diseases especially wasting diseases such as tuberculosis, carcinoma ulcerative colitis and liver cirrhosis are associated with an absolute reduction in total protein but the most marked hypoproteinaemia is found in the nephrotic syndrome Treatment with serum albumin has not however, been found to be of much value in these conditions, the albumin being passed in the urine

The serum globulins are increased in many conditions usually due to an increase in γ globulins which are antibody carriers Hypogammaglobulinaemia however is not common but occurs as an inherited and sometimes an acquired condition It is usually discovered in cases of recurrent infections and is associated with a failure to form the expected iso antibodies The amount of γ globulin present may be determined by electrophoresis or more simply by its ability to inherit anti human globulin serum Purified γ globulin is free of the virus of hepatitis and is used to treat hypogammaglobulinaemia or agammaglobulinaemia keeping the patient free of infection Adult γ globulin containing antibodies against

virus infections such as measles German measles and poliomyelitis is used as a prophylactic measure sometimes but it should be noted that the protection conferred by such preparations is only temporary since the transferred antibody soon disappears from the circulation

TECHNIQUE

Details of the techniques relevant to blood and blood products discussed in the first part of this Chapter are as follows

STANDARD CROSS MATCH

(1) Sterilize cap of blood bottle with iodine in propyl alcohol and withdraw blood in a 2 ml syringe If the bottle supplied is equipped with a pilot tube use blood from the tube [1]

(2) Transfer the blood to a labelled tube and wash well with saline

(3) Set up two wooden blocks the first containing two precipitin tubes one behind the other labelled with the name of the patient and number of the bottle by means of a paper flag inserted in the third hole Behind this flag place two titre tubes (50 mm × 11 mm) In the second block place two tubes as before and at the back of the block two precipitin tubes labelled clearly the first with patient s name and bottle number and the second Coombs control

(4) Place a button of packed washed cells in each of the two titre tubes and the back row tube of the second block

(5) To the first titre tube add sufficient saline to make a 5 per cent suspension by eye and similarly 20 per cent albumin to the second titre tube

(6) With a fine dropping pipette place 1 drop (0 03 ml) of saline suspension in the front tube of the first and second blocks

(7) Rinse the pipette and place 1 drop of albumin suspension in the second tube of first and second blocks

(8) Add 2 drops of patient s serum to each of the precipitin tubes and 5 drops to that containing a button of packed cells

(9) To the Coombs control add a button of washed packed Group O Rh positive cells and 5 drops of a weak incomplete anti D serum

(10) Mix all tubes by flicking with the finger cap with short flat bottom tubes and incubate 2 hours the first block at room temperature and the second at 37 C

(11) Read macroscopically and microscopically

N B (1) Auto agglutinin control is set up in the same manner at room temperature and 37 C (2) The indirect Coombs test may be read after 1 hour at 37 C (3) For special work the cells are trypsinized and then the same procedure followed

[1] It would seem to the author that from a strict medico-l g l point of view the use of a p lot bottle s wrong since t cannot be sa d that the blood in the ma bottle s ompat bl unless t has been tested Howe er the use of the pilot bottle does prevent infect on being introduced into the main bottle of blood and this is a major consideration.

As an added precaution a smear is made from the blood and stained by Gram s method Only blood containing very large numbers of bacteria shows evidence of infection by this means and the use of the pilot tube invalidates the procedure completely

ALBUMIN REPLACEMENT TECHNIQUE

This technique differs from the standard cross match already described only in respect of the albumin tube

(1) Set up the cross match as in previous technique except that two saline suspension tubes are used instead of one saline and one albumin
(2) Incubate 1½ hours at 37 C then gently remove supernatant from one of the saline tubes
(3) Gently add 30 per cent ox albumin in a volume equal to the dis carded supernatant without disturbing the sedimented cells
(4) Incubate a further 30 minutes and read

ALBUMIN ADDITION TECHNIQUE

This method is more suitable for some sera and avoids the removal of saline serum mixture

(1) Proceed as in the albumin replacement technique but instead of removing the supernatant gently add an equal volume of 30 per cent albumin
(2) Incubate a further 30 minutes and read

N B These two modifications of the standard cross match are recommended for two reasons Firstly they are undoubtedly more sensitive and secondly they avoid rouleaux formation

CROSS MATCH WITH NEUTRALIZED MATERNAL SERUM

(1) To 1 volume of maternal serum add an equal volume of the appro priate boiled saliva
(2) Leave at room temperature 30 minutes
(3) To 0 03 ml volumes of the treated serum in two tubes add an equal volume of 2–5 per cent suspension of A_1B cells in saline
(4) Leave for 1½ hours at room temperature and add 1 volume of 30 per cent albumin to one tube
(5) Leave a further 30 minutes and read

N B Should this treatment fail to neutralize the antibodies as shown by agglutination in stage (5) repeat with 2 volumes of saliva and test again

EMERGENCY CROSS MATCH

(1) Wash cells and set up saline and albumin tests as in previous technique

(2) Set up indirect Coombs test
(3) Incubate saline and albumin tubes at room temperature and 37 C for 30 minutes
(4) Leave indirect Coombs test as long as possible, certainly not less than 20 minutes
(5) Centrifuge saline and albumin tubes at 1 500 r p m for 1 minute
(6) Read microscopically

N B Agglutination may only be minimal after 30 minutes centrifuging is of great assistance but agglutinates may break up easily

CONCENTRATED CELL SUSPENSION

The apparatus consists of two long needles connected by rubber tubing to a Y piece and thence to a nylon filter set in a 6 inches × 1 inch tube A shorter tube is set in the filter and connected to a short needle Three air filters attached to short needles complete the apparatus which is sterilized in one container

(1) Without disturbing the sedimented cells sterilize the bottle caps with iodine in propyl alcohol and make entry with the two long needles one to each bottle taking the needle to the bottom of the bottle
(2) Pierce the sterilized cap of a dry sterile bottle with the short needle attached to the nylon filter
(3) Pierce each bottle cap with an air filter and attach that on the dry bottle to a suction pump
(4) Aspirate the cells from each bottle in turn clipping off the first by means of the screw clamp when aspiration is complete

N B The two bottles should be of the same ABO and Rh group and a sample of the mixture withdrawn and examined for haemolysis and clumping The latter may occur should one bottle contain a rarer anti body for an antigen in the second

ESTIMATION OF γ GLOBULIN

(1) Prepare eight dilutions of normal serum in saline over the range of 1 3 000 to 1 10 000
(2) Prepare dilutions of patient s serum in saline over the range of 1 50 to 1 1 000
(3) Dilute anti human globulin serum to optimum titre and add an equal volume to each tube of serum dilution
(4) Test the contents of each tube against red cells sensitized with a strong albumin anti D serum on a tile

The concentration of normal serum γ globulin is assumed to be 1 000 mg per 100 ml Then unknown γ globulin concentration is found from the equation

$$\frac{x}{y} \times 1\,000 = \gamma \text{ globulin in mg per 100 ml}$$

where x is dilution of patient s serum which inhibits the reaction and y is dilution of normal serum which inhibits the reaction

FRACTIONATION OF HUMAN PLASMA (KEKWICK AND MACKAY 1954)

The fractionation procedure is carried out aseptically and solutions of the final products filtered by Seitz ampouled and freeze dried

TREATMENT OF PLASMA

(1) Collect blood into trisodium citrate (100 ml 3 per cent trisodium citrate made up to 540 ml with blood) The blood must be not more than 24 hours old
(2) Separate by centrifuging and syphon off the plasma
(3) Cool to 0–2 C and filter through paper pulp at this temperature

Separation of fibrinogen

(1) Keeping the plasma at 0 C add 11 volumes of ethyl ether
(2) Centrifuge so that the sludge which is precipitated is packed firmly (*see* product (A) below)
(3) Suspend the precipitate in a suitable volume of citrate saline to which has been added 8 volumes per cent of ether and centrifuge again (*see* product (B) below)

The first product analyses as 50 per cent fibrinogen the main con taminant being albumin The second product analyses as 85 per cent fibrinogen

Separation of prothrombin and thrombin

(1) Adjust the supernatant from the fibrinogen separation to pH 5 35 at 0 C using 0 5 M acetic acid
(2) Centrifuge and suspend the precipitate at 0 C in a volume of dis tilled water equal to 40 per cent of the volume of supernatant
(3) Recentrifuge This process removes antithrombin
(4) Suspend the washed precipitate in citrate saline (10 per cent of the original volume of fibrinogen supernatant)
(5) Adjust to pH 7 0 with 0 2 M NaOH and convert to thrombin by the addition of optimal amounts of calcium chloride and thrombo plastin
(6) Stir the mixture vigorously at 27 C
(7) Centrifuge and discard the small amount of sedimented fibrin

The supernatant contains thrombin to the extent of 200μ/ml and activity of the order of 20μ/mg protein

Gamma globulin

(1) Adjust the supernatant from the prothrombin precipitation to pH 5 5 with 0 5 M $NaHCO_3$

(2) Set up indirect Coombs test
(3) Incubate saline and albumin tubes at room temperature and 37 C for 30 minutes
(4) Leave indirect Coombs test as long as possible certainly not less than 20 minutes
(5) Centrifuge saline and albumin tubes at 1 500 r p m for 1 minute
(6) Read microscopically

N B Agglutination may only be minimal after 30 minutes centrifuging is of great assistance but agglutinates may break up easily

CONCENTRATED CELL SUSPENSION

The apparatus consists of two long needles connected by rubber tubing to a Y piece and thence to a nylon filter set in a 6 inches × 1 inch tube A shorter tube is set in the filter and connected to a short needle Three air filters attached to short needles complete the apparatus which is sterilized in one container

(1) Without disturbing the sedimented cells sterilize the bottle caps with iodine in propyl alcohol and make entry with the two long needles one to each bottle taking the needle to the bottom of the bottle
(2) Pierce the sterilized cap of a dry sterile bottle with the short needle attached to the nylon filter
(3) Pierce each bottle cap with an air filter and attach that on the dry bottle to a suction pump
(4) Aspirate the cells from each bottle in turn clipping off the first by means of the screw clamp when aspiration is complete

N B The two bottles should be of the same ABO and Rh group and a sample of the mixture withdrawn and examined for haemolysis and clumping. The latter may occur should one bottle contain a rarer anti body for an antigen in the second

ESTIMATION OF γ GLOBULIN

(1) Prepare eight dilutions of normal serum in saline over the range of 1 3 000 to 1 10 000
(2) Prepare dilutions of patient s serum in saline over the range of 1 50 to 1 1 000
(3) Dilute anti human globulin serum to optimum titre and add an equal volume to each tube of serum dilution
(4) Test the contents of each tube against red cells sensitized with a strong albumin anti D serum on a tile

The concentration of normal serum γ globulin is assumed to be 1 000 mg. per 100 ml Then unknown γ globulin concentration is found from the equation

$$\frac{x}{y} \times 1\,000 = \gamma \text{ globulin in mg per 100 ml.}$$

where x is dilution of patient s serum which inhibits the reaction and y is dilution of normal serum which inhibits the reaction

FRACTIONATION OF HUMAN PLASMA (KEKWICK AND MACKAY 1954)

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(6) Stir the mixture vigorously at 27 C
(7) Centrifuge and discard the small amount of sedimented fibrin

The supernatant contains thrombin to the extent of 200μ/ml and activity of the order of 20μ/mg protein

Gamma globulin

(1) Adjust the supernatant *from the prothrombin precipitation to* *p*H 5 5 with 0 5 M $NaHCO_3$

(2) Reduce the ionic strength by diluting each volume of adjusted supernatant with 3 volumes of distilled water at 0 C
(3) Raise the ether content to 18 5 volumes per cent and lower the temperature to − 4° C
(4) Centrifuge and dissolve the precipitate which contains β and γ globulins in a volume of acetate phosphate buffer pH 4 0 at 0 C The volume used is equal to 45 per cent of the volume of super natant taken
(5) Adjust to pH 5 0 ± 0 02 with acetate phosphate buffer pH 6 0
(6) Add distilled water to lower ionic strength to 0 010 and ether to 9 volumes per cent at 0 C
(7) Centrifuge and discard sediment
(8) Adjust the pH of the supernatant to 6 7 ± 0 05 and raise ether level to 18 volumes per cent with simultaneous cooling to − 3 5 C

The final precipitate analyses as 93–95 per cent γ globulin (*see* (C) below)

Albumin

(1) Precipitate the total protein in the supernatant resulting from the crude separation of globulins by adjusting to pH 5 0–4 8 with 0 5 M acetic acid followed by the addition of 50 per cent ethanol to a final concentration of 25 per cent and lower temperature to − 4 C
(2) Extract the precipitate with a volume of distilled water at 2 C equal to 25 per cent of the volume of plasma from which the precipitate was obtained
(3) Centrifuge and dilute the clear supernatant with an equal volume of water
(4) Adjust pH to 5 1 with 0 5 M $NaHCO_3$ and bring ether concentration to 10 volumes per cent at 0 C
(5) Remove the supernatant from the tacky precipitate without cen trifuging and adjust to pH 4 8 with 0 5 M acetic acid
(6) Bring ether level to 18 5 volumes per cent at 3 5–4 C

The precipitate formed analyses as 98 per cent albumin

(A) This product is used for fibrin foam
(B) This fibrinogen fraction contains AHG and is used for the treatment of haemophilia and together with thrombin for skin grafting and nerve sutures
(C) This γ globulin is pure enough for the treatment of measles

CHAPTER 22

BLOOD SUBSTITUTES

TYPES, USES AND PROPERTIES

INTRODUCTION

IN TIME OF WAR large scale civil disaster or even simple emergencies blood supplies may be inadequate to treat the casualties and a substitute must be employed Perfusion of crystalloid solutions to maintain blood volume results in the tissues becoming oedematous, since a colloid osmotic pressure to balance blood pressure is necessary to control the passage of fluid across the capillary membrane Such an osmotic pressure is provided by the plasma proteins hence plasma and serum are suitable blood volume expanders However, even physiological products such as these are *not perfect although they have a special place in therapy*

HISTORY

Many materials have been tested as blood substitutes some under field conditions where undesirable effects were not immediately observed Gum acacia in saline was used for many years after its introduction in 1917, but eventually it was shown that the gum was stored by reticulo endothelial tissue Methyl cellulose and pectins also share this undesirable property being stored in liver, spleen, kidneys and lungs Isinglass 7 per cent in saline was used at one time but traces of fish protein in impure preparations rendered the material antigenic *Gelatin although valuable by reason of its* protein nature is not used because of the difficulty of keeping the material fluid Some gelatin samples have proved actually dangerous in use Peristan a German preparation containing 2–3 per cent of a synthetic colloid was extensively used during World War II but its use declined for no published reason The plasma volume expander in use today is dextran, which is a collective name for a series of polyglucoses having a high dextrorotation

PROPERTIES OF BLOOD SUBSTITUTES

The ideal blood substitute has yet to be discovered and no plasma volume expander yet known possesses all the attributes necessary

for it to replace plasma The perfect substitute should exert the same osmotic pressure as plasma and preferably be of the same viscosity It should be fluid at low temperatures and be stable under conditions which would cause denaturation of proteins so that it can be sterilized by autoclaving After sterilization the product should be clear so that any deterioration is visible to the eye The material should be non antigenic exert no toxic effect and transmit no disease or allergy It is important that it is only slowly destroyed in the body so that it is retained in the blood stream until its place is taken by normal plasma proteins Once destroyed however, the material should be rapidly and completely eliminated from the body

DEXTRAN

Most dextran is produced by fermentation of sucrose by *Leuconostoc mesenteroides* and *Leuconostoc dextranicum* The cultures are inoculated into large tanks of medium containing sucrose and after 48 hours the viscous dextran is precipitated as a gum by the addition of alcohol The dextran is then hydrolysed with sulphuric acid and heat to break the molecules down to the same size as the plasma proteins

Dextran is used as a 6 per cent solution in saline In many ways it is a suitable plasma volume expander restoring blood pressure and circulatory volume the effect declining slowly over a period of days The material disappears completely from the body being metabolized although the exact manner of metabolism is in doubt Allergic reactions have been reported after its use and some types of clinical dextran have been shown to be antigenic The bleeding time may be prolonged if large quantities of dextran are infused although this effect varies with the recipient It should also be noted that excessive quantities of dextran have an anti coagulant effect The erythrocyte sedimentation rate is sharply increased since dextran has the property of causing intense rouleaux formation This effect may cause difficulty in grouping and cross matching Dextran is easily removed from red cells by washing but sera containing a large concentration of the material used for cross matching may cause errors An anti globulin technique should be used in such cases

FLUID PLASMA AND SERUM

Plasma and serum in their natural state are rarely used today However fresh plasma and serum are sources of anti haemophilic globulin and Christmas factor respectively and may be used to

cover operative procedures on patients affected by the disorders associated with deficiency of these factors

Fluid plasma not required for special purposes keeps well for 12 months in a dark room at 14–20° C Direct sunlight causes denaturation of the proteins and refrigeration encourages clotting Citrated plasma is normally a yellow or orange tinted clear fluid but on storage a deposit of fibrin flakes appears and sometimes actual clotting occurs It is not easily transportable since shaking encourages clotting and bottles should be well filled to avoid this difficulty Clotting occurs during and after Seitz filtration unless the plasma has been treated with ether at − 25° C or repeatedly adsorbed with kaolin

Fluid serum may be taken from clotted blood, or obtained by recalcifying citrated plasma It is stored as fluid plasma or may be refrigerated It has the advantage of not clotting and may be sterilized by Seitz filtration without trouble

DRIED PLASMA AND SERUM

The difficulties inherent in the production and storage of fluid plasma and serum are avoided by the use of dried material Desiccation by heat causes denaturation of the proteins and the use of chemical desiccants results in the production of a large crystal material liable to denaturation and difficult to reconstitute Low-pressure evaporation produces a copious frothing which is avoided by drying *in vacuo* from the frozen state

Serum and plasma for drying are pooled from time expired blood and donors bled especially for the purpose The pools (*see* below) are carefully arranged to avoid spreading of homologous serum jaundice and to avoid high titre antibodies The sterile material is dispensed in standard M R C bottles in 400 ml amounts and shell frozen by spinning the bottles on their vertical axes at 750 r p m in a current of air in a cold room at − 21 C This process leaves a thin layer of serum or plasma frozen against the wall of the bottle Drying is carried out in two stages, firstly by placing the frozen material in a drying chamber which is evacuated to a pressure below 0 2 mm Hg Individual heating of each bottle electrically provides the latent heat of evaporation, the water being condensed on refrigerated coils in the drying chamber The bottles are capped with gauze and cotton wool allowing the passage of water vapour but preventing the access of bacteria After 48 hours the bottles are transferred to a secondary desiccator containing phosphorous pentoxide and after a further 24 hours the bottles are capped with the usual perforated metal tops A hypodermic needle

the febrile attack may be only transient and yet be a sign of a more severe possibly haemolytic reaction The patient s temperature therefore should be recorded every half hour during the transfusion and for 3 hours afterwards In the typical febrile reaction the temperature starts to rise during the transfusion or shortly afterwards and may reach 104° C but latent cases in which the temperature rise is delayed for 24 hours have been recorded The speed of transfusion is unimportant provided the blood does not contain pyrogenic material but should it do so the time at which pyrexia occurs may be affected Thus a rapid rate of transfusion of such blood produces a rise in temperature within 60 minutes of the end of transfusion while a slow rate may delay the rise to occur 2–3 hours afterwards Febrile reactions in children are unaccompanied by rigor and anaesthetized patients may undergo severe reactions unnoticed during the operation

The materials responsible for febrile reactions are nitrogenous products of bacteria and are termed pyrogens They seem to behave as living cells in that they increase in amount on storage but this is due to breakdown to more potent pyrogenic material Pyrogens are adsorbed on to activated charcoal or asbestos so they may be removed from a fluid by shaking with the charcoal with subsequent filtration through sintered glass or preferably by Seitz filtration which both removes pyrogens and also sterilizes To avoid contamination with pyrogens it is essential that distilled water is prepared in a still fitted with splash head run into scrupulously clean glassware and sterilized by autoclaving immediately Should water be allowed to splash over into the condensing system pyrogens will be carried over and if not sterilized immediately the distilled water will allow the growth of bacteria with resultant pyrogen formation

The presence of pyrogens is shown by the rise in rectal temperature of rabbits produced in response to intravenous injection of the suspect material Distilled water under test is rendered isotonic with sodium chloride It is not easy to prove that the giving apparatus is responsible for a pyrogenic reaction so that in the absence of a suitable method of testing it should be ensured that all glass and rubber parts are thoroughly cleaned rinsed with distilled water dried and sterilized immediately Only in this way can the set be said to be non pyrogenic

Pyrogenic reactions are usually controlled by keeping the patient warm during the transfusion and the administration of aspirin Sometimes anti histamines are effective in reducing the temperature

HAEMOLYTIC REACTIONS

The signs and symptoms of haemolytic shock are produced by the transfusion of antigens for which antibodies already exist in the recipient s plasma, by transfusion of antibodies for which antigens are already present, or by the use of haemolysed blood Grossly infected blood without haemolysis may produce the same clinical picture The patient complains of a feeling of heat throbbing in the head, general tingling, breathlessness and lumbar pain Abdominal pain, nausea and vomiting are less common symptoms In the beginning the face is flushed and the pulse full and bounding but these symptoms soon disappear to be followed by rigor and profound collapse Jaundice of mild degree may develop after 24 hours and the patient may become anuric, a condition which if not relieved in 10–14 days will cause death in uraemia This is the clinical picture of a gross haemolytic reaction but minor degrees of haemolysis may pass undetected The so called inapparent haemolysis due to a weak antibody is only recognized by a failure of the haemoglobin level to rise after transfusion Investigations will then reveal the true nature of the red-cell destructive process

Haemolytic reactions following blood transfusions follow the pattern of the episodes of haemolytic anaemia Haemoglobin is liberated into the plasma becomes bound to plasma albumin to form methaemalbumin broken down to bilirubin or excreted in the urine where it often presents as methaemoglobin The plasma from a case of haemolytic reaction taken within a few hours will show free haemoglobin and perhaps clumped red cells and after 24 hours methaemalbumin and an increased amount of bilirubin If any urine is passed it may contain red cell casts and free haemo globin and methaemoglobin The *alpha* band of methaemoglobin will not be seen if the urine is alkaline

INVESTIGATION PROCEDURE

The investigation of a blood transfusion reaction (Fig 45) begins with the immediate termination of the drip If it can be shown that the reaction is allergic or febrile due to the patient s condition rather than a fault in the blood then the transfusion can continue with appropriate medication Should the evidence suggest a haemolytic reaction the drip is discontinued and the remainder of the blood and the set sent to the laboratory At the same time venous blood is taken from the patient into heparin and also a dry sterile bottle It is essential that care be taken to ensure that the blood is not haemolysed by rough handling The site for venepuncture

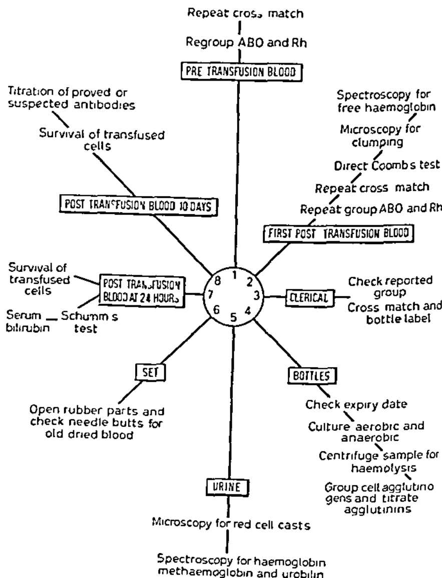


FIG 45—Scheme of investigation of a blood transfusion reaction The plan details the order in which investigations should be carried out It is assumed that the reaction is febrile or haemolytic allergy having been excluded the only other investigations necessary being pyrogen check on same batch of ACD (inconclusive) and check of grouping sera

should be away from the drip site a large needle used and the minimum of suction exerted The ward must be instructed to send all urine to the laboratory and enquiries made regarding the treatment received by the blood since it left the blood bank It may be found that the blood has been kept in contact with the freezing chamber in the ward refrigerator, left at room temperature for a considerable time or even warmed up' before being administered Difficulty in setting up the drip may have resulted in the bottle being opened, then sealed up again and left for some hours before another attempt has been made Any information which can be gleaned from the nursing and medical staff may be valuable in deciding the aetiology of the transfusion reaction

The repeat group and cross match on the pre transfusion blood sample excludes technical and clerical errors when combined with those results shown in the daybook Apparent compatibility with the post transfusion sample is not definite proof that a haemolytic reaction has not taken place since the antibody may have been absorbed by the transfused blood or that the methods used are failing to detect the antibody All methods including enzymes must be included in the repeated cross match A negative cross matching could also be given if high titre antibodies donated by the transfused blood have caused lysis of the recipient s cells The direct Coombs test if positive indicates sensitization of the cells by antibody and microscopical clumping of a 2 per cent suspension of the first post-transfusion sample is added confirmation of the serological nature of the reaction In a very carefully taken sample of blood haemolysis visible to the naked eye may be obvious but if not spectroscopical examination at this stage is of value

Since most transfusion reactions of a haemolytic nature are clerical it is essential that human errors are checked The daybook entries must be checked with the pre transfusion reports and the labels on the bottles examined to ensure that the correct details of the patient are given The expiry date of the blood is also rechecked The finding of free haemoglobin in the supernatant of the centrifuged sample from the blood bottle indicates damage to the red cells since leaving the laboratory It has been known for blood to be grossly mistreated in operating theatres and wards a classical example being that of a nurse who sterilized the blood in a fish kettle sterilizer The usual type of organism found in contaminated blood is a coliform which can utilize citrate and exist and even multiply at 4° C Although haemolysis of the blood is usual it is not invariable even when the blood is teeming with organisms It must be rare indeed that a bottle of blood is sent from the National

Blood Transfusion Service wrongly grouped but the possibility must be borne in mind when investigating a reaction The possibility of high titre antibodies particularly where Group O blood is used for a recipient of another group must also not be overlooked

The presence of red-cell casts in the urine is not conclusive evidence of a haemolytic reaction Such an appearance may be seen in acute nephritis and it is worth remembering in this connexion that a positive Schumm s test on the urine can be obtained in a haemolytic reaction in a subject who has severe renal disease Excess of urobilinogen is shown by means of Ehrlich s reagent and also shown spectroscopically as urobilin after oxidization with iodine

Small dried clots from previous transfusions can by their pyrogen content cause a febrile reaction The usual sites for such material are in the rubber tubing and the needle butts The tubing must be *cut longitudinally and laid open to detect the dried crusts and the* needle butts scraped

Serum bilirubin reaches its highest level 24 hours after a haemolytic reaction and methaemalbumin instead of haemoglobin is present in the plasma The second post transfusion blood sample is therefore of the greatest importance in deciding that such a reaction has taken place Differentiation should be made between direct and indirect bilirubin especially where there is liver disease The survival of transfused cells can only be estimated if there is a known blood group incompatibility and Ashby s technique can be used here

The final sample of blood from the patient taken 10–14 days post transfusion may be used to titrate the antibody responsible for the reaction or to check on any rise in titre of antibodies known to be present thus tending to prove which one was responsible The survival of transfused red cells if of a different blood group from the patient may be estimated on this sample and at further intervals

IDENTIFICATION OF ANTIBODIES

If the full blood group genotype of the patient is known the identification of the antibody responsible for the transfusion reaction may be a simple matter since it is possible to state what antibodies he can make In the majority of cases however all that is known in the ABO and D groupings and in such cases a wider investigation is often necessary Gross incompatibilities are usually ABO but there are exceptions—some cases being mild and severe reactions occurring with other groups It may be necessary in order to prove ABO incompatibility to show that the antibody present is immune in nature

TABLE XVIII

RESULT OF USING A PANEL OF CELLS

Known cells	37° C		16° C		4° C		ICT	ABO	Rh	MN	S	P	Lu^a	K	Le^a	Le^b	Fy_a
	Sal	*Alb*	*Sal*	*Alb*	*Sal*	*Alb*											
A	−	−	−	−	−	−	−	O	R_1R_1	MN	+	+	−	−	−	+	+
B	−	−	−	−	−	−	−	O	R_1r	N	−	+	−	−	−	+	+
C	−	−	−	−	−	−	−	O	$R'r$	MN	−	−	−	−	−	+	−
D	−	−	−	−	−	−	−	O	$R''r$	MN	+	+	−	−	+	−	−
E	−	−	−	−	−	−	−	O	rr	M	+	+	−	+	−	+	−
F	−	−	−	−	−	−	−	O	R_1R_1	M	+	+	−	+	−	+	−
G	+	−	+	−	−	−	−	O	R_1r	N	−	+	+	−	+	−	+
H	+	−	+	−	−	−	−	O	R_1^wr	MN	−	+	+	−	+	−	+

The antibody can be only anti Lu^a so far as the groups tested are concerned

Immune anti A and anti B differ from the naturally occurring antibodies in several ways First, immune anti A and anti B have a higher titre at 37° C than at 16° C so that if duplicate titrations are set up at these temperatures and the titre is the same or higher at 37° C the antibody is regarded as immune The titre of immune ABO sera is enhanced by serum but depressed by ox albumin so that if the immune titre is higher than the natural agglutinin titre it can be made obvious by titration in AB serum

If fresh serum is used the presence of complement causes lysis with the immune antibody and this characteristic may also be used to differentiate the antibodies Finally the serum may be partially neutralized by group specific substance Persistence of agglutinating power in the serum diluted in compatible serum is regarded as indicating an immune antibody

Preliminary screening with O R_1R_2 cells will give an indication that an Rh antibody is the cause of the reaction the identity being established by the procedure detailed in Chapter 17

Further identification of antibodies simply involves using a panel of cells Group O rr but containing the other blood group antigens between them Thus the other antigens represented should include those of the MNS, P Lutheran Lewis Kell, Duffy and Kidd groups and tests should include saline and albumin tests and anti globulin reactions It may be necessary later to extend the scheme to include enzyme techniques but the possible causes of the reaction should have been whittled down to a very few suspects by this time

A typical result of using a panel of cells is shown in the protocol (Table XVIII)

CHAPTER 24

SEROLOGICAL LABORATORY AND BLOOD BANK ORGANIZATION

ESSENTIAL REQUIREMENTS AND RECOMMENDATIONS

GENERAL CONSIDERATIONS

It is an unfortunate fact that blood transfusion technique has grown up as a part of laboratory work generally, rather than a separate subject This means that in many hospitals serological work has to be carried out in confined and generally unsuitable surroundings The ideal blood bank and serological laboratory should be a self-contained unit in reasonable proximity to the casualty department theatres and wards The rooms should be deliberately arranged so that access to the laboratory and blood bank is through the office ensuring that unauthorized visitors are checked before they can invade the privacy so essential for the mental concentration needed in this work In this respect the secretary must be fully cognizant of what is being done at any particular moment since all telephones should be in the office where also specimens are received The unit should consist of the office blood bank cold room serology laboratory and sterile room and wash up and sterilizing department If large scale bleeding of donors is contemplated reasonable provision must be made for this but as a rule only the individual donor attends the laboratory In this case it is useful to have a small examination room where the donor may be bled and allowed to rest after the donation

THE SEROLOGY LABORATORY

The general requirements of a serological laboratory conform to those of other medical laboratories There should be good natural light along the benches which may be of hardwood or plastic surfaced Since little staining work is done only one small sink is required for this purpose It is useful however to have one larger sink where a supply of hot water may be obtained and also where a water pump can be installed A wash hand basin is a normal requirement of all laboratories The lighting if of the strip variety

should be at optimum height over the benches which should also be equipped with lamps of the Anglepoise type Large water baths one each at 37° C and 56° C, should be on the bench and a smaller portable type is useful

It is recommended that each technical worker should have a microscope and small centrifuge for his own work A larger centrifuge should be on its own small bench to avoid any vibration disturbing microscopical observations The height of the working benches is a matter of individual choice but in the author s experience table height benches and comfortable chairs are much preferable to the high benches and stools of other types of laboratory

Incubators at 16° C and 37° C are necessary in this laboratory but should not occupy bench space It would be convenient if such fittings could be fixed to the wall at a reasonable height rather like kitchen cupboards Three refrigerators are essential one being of the ice-cream conservator type thermostatically controlled at — 20° to — 30 C The other refrigerators should be of household type at 4° C but without a *freezing chamber* The first is necessary for reagents (cells sera etcetera) for day to day working and opened bottles which are under investigation The other refrigerator is for blood which is being cross matched and freshly collected blood which has yet to be checked for its group and other details

Storage of tubes

It is recommended that tubes be kept in tinned boxes which are dust free and stored in the bench cupboards rather than in drawers Wood blocks and other test tube racks are also stored in the cupboards Pipettes should be kept in the tins normally used for the purpose

Use of benches

The benches should always be clear of apparatus and materials except that actually being used The only other materials necessary on the bench should be a discard tray and a deep bowl of tap water in which to immerse in bundles the tubes no longer required Syringes if used for puncturing blood bottles should be kept in a top drawer on the working bench

THE STERILE ROOM

A small part of the serology laboratory should be completely partitioned off by a glass wall The door should be close fitting and ventilation produced by a means which includes a filter The bench should be equipped with a sink and gas taps Also in this room is

the refrigerated centrifuge which can take MRC bottles The bench should preferably be plastic topped so that it can be cleaned down with antiseptic, and a means provided of spraying the room with a bactericidal aerosol This room should be used only for sterile procedures such as packing cells, washing cells and removal of plasma for processing

THE BLOOD BANK

The blood bank should consist of a small room adjoining the serology laboratory, again being accessible only through the office It should contain a refrigerator of household type or open top ice-cream type operating at + 4° C and plainly marked to the effect that it contains only blood which has been cross matched On the wall or even on the refrigerator door should be a board like a letter rack In the rack there should be a card for every bottle of blood in the refrigerator The card must have the name ward index number, group, numbers on blood bottles and date of cross match A space should be left for the signature of the person receiving or removing the blood from the refrigerator together with the date and time it was taken After signing the card the responsible person should then ' post it in a box provided It should be a rule that after hours only a medically qualified individual should have access to the blood bank

A similar card system should be attached to another refrigerator containing O Rh negative blood and plasma This refrigerator should be marked plainly *Emergency Only* and only the gravest emergency should require the blood stored here

A third refrigerator at − 20° C should also be available for the storage of fresh frozen plasma and the only other materials in this room need be stocks of giving sets

COLD ROOM

The bulk of the blood supplies should be in an actual cold room at + 4° C Access to such supplies should of course be denied to all but the staff of the unit Bulk stocks of albumin and other refrigerated reagents should also be kept in this room

WASH UP AND STERILIZING ROOM

The functions of the wash up room are rather more complicated than simple washing of glassware since it should also be a preparation room Distilled water in bulk should be prepared here in a still of the Manesty type fitted with baffles to prevent carry over and a

bench should be available for the preparation of pipettes packing of giving and taking sets and dispensing of anticoagulant in blood bottles A drying oven hot air sterilizer and autoclave are essentials The washing up should be done in large sinks and the bottom of the sink and draining boards covered with plastic to minimize breakages Large gas rings should be available for boiling bowls of precipitin and titre tubes

RECORDS

Blood transfusion records begin with the arrival of blood from the N B T S centres A book should be kept in which the group and index number of each bottle is entered together with its disposal and comments regarding reaction A card index system arranged alphabetically must be kept of all patients, but the only entry on the card should be dates of laboratory tests This file is kept in the office and the relevant card brought to the serological laboratory by the secretary together with specimens of blood when they arrive The record book kept by the technicians contains columns for the patient s details together with details of all reactions obtained when grouping and cross matching The last column is for the signature of the technician concerned who should also enter the date on the patient s card This daybook is the most essential record of the department and should never leave it Details of results of tests must be entered straight into the book and not copied from scraps of paper

REQUEST FORMS

Requests for blood or serological investigations should be made on a form which includes full obstetric details and history of trans fusions and should not be accepted unless these details are given or a reason for not being able to obtain them No report on the blood group or cross matching should leave the laboratory since these details together with a signature should be on the label attached to the blood bottle Ordinary blank report forms may be used for details of serological investigations and blood groups which do not require transfusion that is ante natal cases and so on

ALARM SYSTEMS

Blood is a drug which quickly deteriorates and becomes dangerous to use if storage requirements are not met To avoid disaster it is essential that all refrigerators are fitted with alarms which will

indicate if the temperature is too high or too low Lights as alarms are useless since they may be unnoticed or ignored Bells or buzzers which will ring continually until the fault is rectified should be placed in the porter s lodge with instructions that a responsible person be immediately notified The more strident the bell the more speedily will the individual be called

CHAPTER 25

USES OF STATISTICS

APPLIED MATHEMATICS IN BIOLOGICAL SCIENCE

INTRODUCTION

THE SCIENCE of statistics is a branch of applied mathematics used to extract information from numerical data It is of great value in the biological sciences where it is used to estimate errors arising in apparatus and technique, and to determine whether a series of experimental results are significant or due to chance In this latter respect it is not always possible to be dogmatic but to be able to state that the probability of the results being due to chance is less than 1 in 1,000 is almost as good as a certain answer The application of some statistical methods requires considerable mathematical knowledge but a few valuable techniques may be learned with a minimum of this skill

Data

Data for analysis must be reliable and to make handling easier is arranged in classes but it must be remembered that much is lost if the classes are too wide in scope The data is expressed in equations using symbols usually letters of the Greek alphabet but these are always used by definition or have meanings which are accepted by other workers

DEFINITION OF TERMS

Statistics treats of an infinitely large series of measurements termed a population but for practical purposes smaller numbers or random samples are taken The analysis of these samples yields results which bear a relationship to the whole but since variable chance factors operate it will be obvious that the larger the sample the more representative the results The data may deal with the presence or absence of an attribute for example haemoglobin S in Turks or a variable for example red cell diameter In the simplest case of an attribute only two classes are formed but an attribute

can become a variable if, for example, the abnormal haemoglobin investigation treats of the amount of sickle haemoglobin in Turks The number of observations in a class is termed the class frequency or simply frequency, and the difference between classes, the class interval

Calculation of mean value

If a mean value is calculated for a biological random sample, the chance factors operating equally in a positive and negative fashion produce individual values distributed about the mean in such a manner as to make a symmetrical bell shaped curve—this is termed the normal distribution Other types of distribution are known and all are defined by constants termed parameters The normal distribution is characterized by the parameters mean and variance The mean (arithmetic mean) is the sum of all individual values divided by the total number (N) and the variance is the sum of the squares of the deviation of the individual values from the mean divided by the total number of individual values The standard deviation (mean quadratic deviation) is the square root of the variance Where variation is to be expected the mean has no real value without the standard deviation The deviations from the mean are squared in the calculations to eliminate minus signs which would tend to produce zero where positive and negative chance factors are operating equally The shape of the curve in the normal distribution is a function of the standard deviation a smaller standard deviation producing a steeper narrower curve and a larger producing a flatter curve with a spreading of the base

In a normal distribution frequency curve, 95 5 per cent of all chance variation fall within the range limited by $M \pm 2\sigma$ The probability that a value outside the limits represents a normal deviation which has been included by chance is 100–95 5 per cent = 4 5 per cent A greater percentage than this outside the limits laid down indicates some factor other than chance operating

TESTS OF INDEPENDENCE

If experiments are planned correctly statistics provides a method of testing the agreement between the observed and expected results and thus proof that the hypothesis is correct Probability can be calculated from other distributions but the χ^2 distribution is used a great deal in blood group serology for this purpose From the table of χ^2* we obtain the value P which is the probability that random

* *Statistical Tables for Biological Agricultural and Medical Research* 3 d ed By A Fisher Sc.D., F.R.S and Frank Yates Sc D Edinburgh and London Oliver & Boyd Ltd

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Example

Counting 500 cells

$\sigma = \sqrt{500} = 22$ approx

$\nu = \frac{22}{500} = 4\ 4$ per cent

Counting 1 000 cells

$\sigma = \sqrt{1\ 000} = 31$ approx

$\nu = \frac{31}{1{,}000} = 3\ 1$ per cent

Counting 2 000 cells

$\sigma = \sqrt{2\ 000} = 44$ approx

$\nu = \frac{44}{2\ 000} = 2\ 2$ per cent

SUMMARY

It is not possible in these few pages to give more than a slight introduction to a subject which is of increasing importance in medical science The techniques most valuable to the technologist can be learned quite easily and with practice the classification of experimental results for analysis can be done correctly The remainder of the work is calculations and reference to tables It must not however be imagined that the study of statistics is easy For those with mathematical ability it is a fascinating subject which will fully repay the time spent reading the appropriate textbooks

sampling will give a value of χ^2 as great as or greater than the value actually obtained A comparison of two samples with two or more classes is presented in tabular form and termed a 2 × 2 table χ^2 is calculated and P read from the table, entry being made at the line of the appropriate degree of freedom For 2 × 2 tables the degree of freedom is equal to 1 An example will make this more clear The figures in the 2 × 2 table are those of Race and Sanger for the results of grouping known members of the MN group with anti S

		M + MN	N	
anti S	+	93	15	108
	−	52	30	82
		145	45	190

$$\chi^2 = \frac{(93 \times 30) - (52 \times 15) \times 190}{145 \times 45 \times 108 \times 82} = 13 \text{ for 1 degree of freedom}$$

This value of χ^2 is not on the table for 1 degree of freedom which means that P is less than 0 001 that is that the probability that the association between MN and S is due to chance is less than 1 in 1,000

RED CELL DIAMETER DISTRIBUTION CURVE

The statistical method of Price Jones has already been mentioned (*see* Chapter 5) but it is relevant to recall some of the features The total number of red cells in the body constitutes the population under investigation and the actual number of red cells measured is the sample The red cell diameter distribution curve is an example of the normal distribution and the standard deviation is a measure of anisocytosis

COUNTING CHAMBER ERROR

The error inherent in counting cells in a haemocytometer has been dealt with in Chapter 3 but must be clarified here The distribution of cells in a counting chamber follows that of a Poisson series that is a distribution characterized by the mean being equal to the variance (σ^2) The calculations enable the error to be found with great accuracy and show quite clearly that the only way to minimize it is to count more cells

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INDEX

INDEX

C

D

I

J

K

R

S

T